ORIGIN AND SEASONAL VARIATION OF BACTERIAL CONTAMINATION OF MILK

A thesis

submitted in partial fulfilment

of the requirements for the Degree

of

Master of Science in Microbiology

in the

University of Canterbury

by

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University of Canterbury

2003

ACKNOWLEDGEMENTS

I was fortunate to have the support of many people during this research and therefore need to express my many thanks to these people.

To Chris, Clive, Steve and Lance for making this research possible.

To Andrew and Tony for your constant encouragement and guidance.

And finally, but most importantly, to my family and friends, thank you for simply being there.

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LIST OF ABBREVIATIONS

CIP	Cleaning in place
CFU/ml	Colony forming units per millilitre
°C	Degrees centigrade
G	Goulds medium
g	Grams
g/L	Grams per litre
homo	Homogenised
kDa	Kilo Daltons
kPa	Kilopascals
LB	Luria bertani medium
µl	Microlitres
MPCA	Milk plate count agar
ml	Millilitres
Min	Minutes
ox-	Oxidase negative
ox+	Oxidase positive
ppm	Parts per million
%	Percentage
SEM	Scanning electron microscope
sec	Seconds
spp	Species
STER	Standard error

ABSTRACT

Type, origin and seasonal variation of psychrotrophic bacteria contaminating milk from a Christchurch milk-processing factory was investigated.

Bacteria were monitored in bottled milk over ten days at 7°C. *Bacillus licheniformis, B. subtilis, Pseudomonas fluorescens* and *Ps. putida* were identified, with populations exceeding $5x10^5$ CFU/ml after five days.

Origin of the bacterial contamination was determined by isolating from milk at various points on the processing line including newly pasteurised, storage tank and pre-filler, as well as filler and bottled milk. Environmental isolations were made from chlorinated water, recycled glass bottles and factory swabs. *Ps. fluorescens* and *Ps. putida* were present in the milk immediately before entering bottles and *Ps. fluorescens* was also isolated from environmental swabs. Additionally, *Bacillus spp* were isolated and included *B. circulans* and *B. cereus* both of which were found in newly pasteurised milk.

Seasonal variation in psychrotrophic bacterial populations were established by comparing bacteria isolated from milk at various sites throughout the milk process line in the summer with those found in winter. The most significant seasonal difference was seen in raw milk which contained high levels of *Pseudomonas spp* in the winter and smaller populations of Gram-positive cocci in the summer. For bottled milk, at the consumer expiry date, increased levels of *Pseudomonas spp* were seen in early summer, mid-autumn and late autumn, but decreased levels were seen in mid-spring.

CHAPTER I

INTRODUCTION

Milk is a complex biological fluid that contains lipids, proteins, carbohydrate material and salts. Milk protein is composed of 27g/L of casein and 4-7g/L of whey proteins. Caseins are grouped according to their amino acid sequence into α_{S1}-, α_{S2}-, β- and κ-casein; in freshly secreted milk they are aggregated in particles referred to as micelles. The model for casein micelle has numerous subunits, κ- casein occupies the surface and α_{S1}-, α_{S2}-, β-casein are located in the interior of the micelle. The lipid content of milk is 37g/L and of this 98% are triglycerides. (Cromie,1992). Lipids are present in a globular form in milk, with an outer membrane consisting of proteins, phospholipids, glycolipids, sterols and glycerides that protect the triglycerides from the aqueous phase. The carbohydrate component of milk is predominately lactose with a concentration of 48g/L. Salt components of milk are numerous but are present in very small quantities with the major cations being calcium, magnesium, potassium and sodium and the major anions consisting of chloride, citrate and phosphate. These salts contribute no greater than 9.2g/L (Robinson, 1990).

This rich source of nutrients provides an ideal medium for microbial growth and consequently there are a myriad bacteria, yeasts and moulds associated with milk products. Many of these organisms that can inhabit milk are potentially harmful to humans and include organisms such as *Listeria monocytogenes*, *Campylobacter jejuni* and *Mycobacterium tuberculosis*, as well as viruses such as Hepatitis A and Cow-pox virus (Robinson, 1990). The pasteurisation of milk involves heating to 73°C for 15 sec and was originally designed to prevent milk products acting as a carrier of these human pathogens (Nickerson and Sinskey, 1972).

The pasteurisation process, when combined with the use of refrigeration in all stages of milk processing, has benefited the diary industry considerably by extending the shelf life of dairy products. The introduction of refrigeration has, however, lead to a change in microbial flora associated with milk. These flora were once dominated by mesophilic bacteria, those with optimal growth temperatures ranging between 25°C and 40°C (Fairbairn and Law, 1986).They now are dominated by psychrotrophic bacteria, which are able to grow readily at temperatures below 7°C irrespective of their optimal growth temperature (Cousin, 1982).

The increase in psychrotrophic bacteria in milk has caused much concern in the dairy industry. A large amount of research has been dedicated to this problem. To gain an understanding of the problem as a whole, however, there is a need to understand the psychrotrophic bacterial dynamics of individual milk processing factories. This thesis will cover the origins and seasonal variations of bacterial contamination of processed milk in a single milk-processing factory in Christchurch, New Zealand.

1.1 THE PROCESSING OF MILK

1.1.1 Milk Pasteurisation

Dairy cows are milked twice daily and the milk is collected from the farm every day from September through to March. From April through to August, however, the milk is stored for up to two days on the farm within refrigerated tanks. The tankers used to collect milk in the Canterbury region are insulated but are not refrigerated. They collect milk from a number of farms before returning to the milk-processing factory. A number of tankers deposit their milk into one of three raw milk silos where it is stored at 4°C for 2-6 hours. From here the milk undergoes the pasteurisation process: this begins at a separator where the cream is removed at a temperature of 55°C. The milk is then pumped to the pasteuriser where it is heated to 73°C for 15 seconds. From here the milk is cooled by the heat exchange system. This system allows pasteurised milk to be cooled from 73°C to 15°C and

in exchange the raw milk is heated from 4°C to 55°C. Once the milk has been brought down to 15°C it is cooled further by being refrigerated to 4°C before entering one of many storage tanks. The now pasteurised milk is stored in these tanks until it is packaged. The time taken for the milk to move from the cream separator to the beginning point of post pasteurisation cooling is approximately 60 seconds and to the storage tanks is approximately 2 minutes.

1.1.2 Milk Bottling

Milk is pumped out of the pasteurised milk storage tanks and flows into a bottle filler bowl until the 'float' contacts the cut-off valve trigger, stopping the flow of milk into the bowl. Recycled bottles are moved by a complex track system that extends from the bottle washer to the bottle filler. When reaching the bottle filler, bottles are placed on to individual pedestals which gradually increase in elevation and force the bottle up into a filler rubber, as shown in Figure 1a. When the glass rim of the bottle contacts the filler rubber, it forces the rubber up, causing a rubber diaphragm to flex and releasing milk from the bowl into the bottle. A venting tube enables the air contained in the bottle to be released and the bottle is topped up to the brim. The pedestal then moves the bottle down away from the filler rubber and the bottle is moved further down the line to be capped, as shown in Figure 1b. The bottle filler contains fifty-six filler nozzles and hence fills fifty-six bottles simultaneously.

1.1.3 Cleaning Regimes

Farm tanks are cleaned every day or second day depending on when they are emptied. The cleaning involves a rinse with water to remove any milk residue, a cleaning rinse with a caustic solution and a sanitizing rinse with chlorinated water. The tankers that are used to transport milk are cleaned in a similar manner; however, once weekly they undergo an acid wash to remove protein build up.

At the milk-processing factory every surface which is in contact with the milk is cleaned. This is done by the 'cleaning in place' system or CIP system. This system delivers a 65°C caustic wash, a 65°C acid wash and numerous cold water rinses between treatments. The final sanitizing rinse delivered by the CIP system is a chlorinated water rinse; the level of water chlorination used at this stage varies between <5ppm to >15ppm. The raw milk tanks, the pasteurised milk tanks and pipelines are all washed with an acid cycle every day, whereas all other tanks and pipelines at the factory undergo this acid wash only once a week. The floors of the packaging rooms are cleaned once a day with an antibacterial foaming agent, as shown in Figure 1c.

Glass bottles are recycled numerous times in the Christchurch region and the cleaning they undergo involves a warm water pre-rinse to remove any milk residue and foreign matter, a series of caustic wash rinses that cycle through different strengths and temperatures, before a cold water rinse and air-drying.

The bottle filler bowl, nozzle and rubbers are cleaned once daily. The filler rubbers are removed, placed on to a wire and along with the filler nozzle are put into an air-agitated wash bath where they undergo a caustic wash. The filler nozzle and rubbers are put back into place and the filler bowl is filled with an iodine/acid solution overnight. The solution is flushed out of the bottle filling system immediately before use and the system is rinsed with water. The first 56 bottles from the first run are discarded to eliminate possible chemical contamination of the milk.

1.2 MILK MICROBIOLOGY

In the past poor refrigeration has been largely responsible for milk spoilage because of the growth of mesophilic bacteria such as the lactic streptococci (Fairbairn and Law, 1986). Since the introduction of refrigeration systems for the storage of raw and post-pasteurised milk there has been a decrease in mesophilic bacterial populations and an increase in psychrotrophic bacteria.

Figure 1a: *Filling of milk bottles.*

Figure 1b: *Capping of milk bottles.*

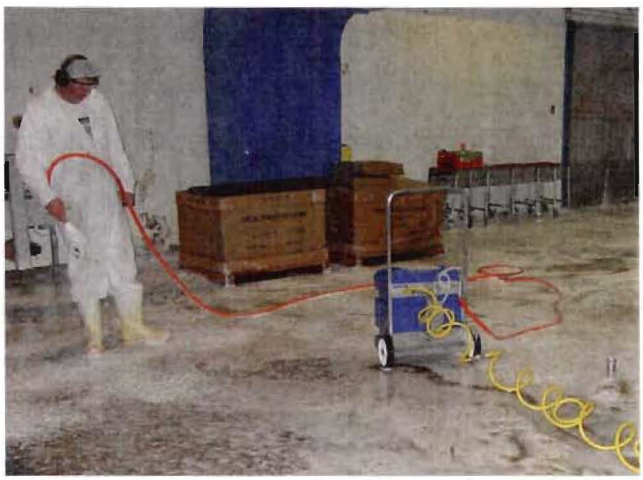

Figure 1c: *Application of antibacterial foaming agent.*

Psychrotrophic organisms contribute to milk spoilage in two different ways. Firstly, they produce lipolytic and proteolytic enzymes which they secrete into the raw milk during storage. Secondly, psychrotrophic organisms make up the majority of the post-pasteurisation contaminant population. Lipolytic and proteolytic enzymes produced by these post-pasteurisation contaminants contribute to shortened shelf lives of pasteurised milk products (Wiedmann *et al.*, 2000).

1.2.1 The Psychrotrophic Community

The psychrotrophic community has yet to be fully characterised but is made up of a very broad group of organisms including bacteria, yeasts and moulds. Bacteria may include Gram-positive and negative rods, cocci and vibrios. These bacteria may be spore formers or non-spore formers, aerobic, facultative anaerobic or anaerobic microbes. The Gram-negative bacteria include species of *Achromobacter, Acinetobacter, Aeromonas, Alcaligenes, Chromobacterium, Citrobacter, Escherichia, Flavobacterium, Klebsiella, Pseudomonas, Serratia* and *Vibrio.* Gram-positive bacteria include species of *Arthrobacter, Bacillus, Clostridium, Corynebacterium, Lactobacillus, Microbacterium* and *Streptococcus.* (Cousin, 1982).

Psychrotrophs of most concern to the dairy industry are grouped into two types, Gram-negative, non-spore forming rods and Gram positive spore forming rods. The first group includes species of *Pseudomonas, Enterobacter* and *Klebsiella.* Of these, *Pseudomonas species* are considered most damaging to the sensory quality of dairy products where spoilage is caused by the prolific production of proteases and lipases (Cousin, 1982). Ralyea *et al* (1998) isolated 233 bacteria from raw and pasteurised milk and of these thirty were identified as *Pseudomonas spp.* The pseudomonads commonly associated with milk are *Ps. fluorescens*, *Ps. fragi*, *Ps. putida* and *Ps. aeruginosa*; all, with the exception of *Ps. fragi*, are fluorescent pseudomonads (Robinson, 1990). Further research by Wiedmann *et al* (2000) concluded that *Ps. fluorescens* represented the predominant bacterial cause of flavour defects in milk.

Pseudomonas spp dominate the raw and post-pasteurisation environment as a consequence of their high growth rate at lower temperatures. The generation time for *Ps. fluorescens* at 7.2°C is 7.2 hours (Bishop and White, 1986). In comparison the generation times of *Bacillus spp* are considerably longer ranging between 22 to 26 hours at 7°C (Sørhaug and Stepaniak, 1997, Cousins, 1982).

When *Pseudomonas spp* are not present, Gram-positive, spore-forming rods dominate the microbial populations of pasteurised milk. These bacteria are considered to be the second most important group of concern to the dairy industry, because of their heat resistant spores as well as proteases, lipases and phospholipases. These bacteria include species of *Bacillus* and *Clostridium*, however, the aerobic environment of bottled milk favours the dominance of Bacillus strains such as *B. cereus, B. circulans, B. licheniformis* and *B. subtilis*. *Clostridium spp,* due to the strictly anaerobic nature of many strains, are considered to be spoilers of cheeses rather than bottled milk (Robinson, 1990).

1.2.2 Natural Psychrotrophic Reservoirs

Pseudomonas spp are isolated from soil and water (Robinson, 1990), however, the entry point into the raw milk is mainly via poor sanitation of dairy equipment. Milk produced on farms under good sanitary conditions contain less than 10% psychrotrophs whereas those under poor sanitary conditions contain up to 75% psychrotrophs (Cousin, 1982) with the majority of these bacteria being pseudomonads.

Bacillus spp primarily inhabit the soil, although they are widely distributed in vegetation, animal hair and fresh water (Roberts and Tompkin, 1996) unlike *Pseudomonas spp* they enter the raw milk via water and fodder or feed (Robinson, 1990).

1.2.3 Effect of Pasteurisation on Psychrotrophic Bacteria

Bacillus spp are thermoduric psychrotrophs which means they are able to survive the high temperatures of pasteurisation and then grow at refrigeration temperatures. They do this by their production of heat resistant spores. *B. cereus* spores, like many thermoduric psychrotrophs, are similar in nature to mesophilic spores, however, they have 15-20 times higher resistance to heat than most mesophilic spores with D values (the time required to destroy 90% of spores) varying between 3 to 19.1 min at 95°C, depending on the strain (Roberts and Tompkin, 1996).

Pseudomonas spp, unlike *Bacillus spp,* do not produce spores and therefore the vegetative cells are susceptible to pasteurisation (Schröder, 1984).

1.2.4 Effect of Psychrotrophic Bacteria on Milk

1.2.4.1 Proteolysis of Milk

Extracellular proteases are produced in great quantities by many *Pseudomonas spp* and in smaller quantities by *Bacillus spp. Pseudomonas spp* are thought to produce either one type of protease or one main protease and trace amounts of no more than two others (Liao, 1998). These enzymes are metalloenzymes with molecular masses ranging between 22 to 50kDa depending on the strain (Cousin, 1982, Liao and McCallus, 1998) and contain one zinc atom and up to eight calcium atoms per molecule (Sørhaug and Stepaniak, 1997).

Proteolytic enzymes cause the development of bitter flavours in milk as well as the clearing and coagulation of milk (Adams et al, 1974). They do this by degrading κ-and β-casein readily and α_{S1}-casein more slowly (Law et al, 1977). Pseudomonad proteases have an optimum activity at 40°C. When temperatures are reduced to 25°C, however, the

enzyme activity decreases to between 60 to 80% of optima (Cousin, 1982) and at 4°C to 7°C the enzyme activity is further reduced to 30% of optima (Cromie, 1992).

The reason for increased protease production by psychrotrophic bacteria in milk at refrigeration temperatures is unknown. Bacteria may produce them in response to an increased availability of proteins in the environment (Liao and McCallus, 1998). The random non-helical structure of casein means that it is highly susceptible to proteolysis and at refrigeration temperatures the level of free β-casein in solution is increased by up to 30% (Cromie, 1992). Alternatively, increased protease production may be an effect of temperature as microbes synthesise increased amounts of enzymes in response to reduced enzyme activity (Cousin, 1982).

1.2.4.2 Lipolysis of Milk

Lipases cause rancid or soapy off-flavours in milk and *Pseudomonas* and *Bacillus spp* are large producers of these enzymes (Bishop and White, 1986). Pseudomonads are thought to produce only one type of lipase which often forms large aggregates or lipase-polysaccharide complexes (Sørhaug and Stepaniak, 1997). The enzymes are activated by pasteurisation due to the dissociation of an enzyme-inhibitor-complex during heating. Lipases hydrolyse the triglycerides into di- and monoglycerides and then into glycerol together with free fatty acids (FFA). The build-up of FFA in milk causes flavour defects. The optimal temperature for lipase activity ranges between 30°C to 50°C, however, the enzymes still retain activity at temperatures as low as −10°C (Cromie, 1992).

1.2.4.3 Phospholipolysis of Milk

Phospholipases are produced by a number of psychrotrophic bacteria including species of *Acinetobacter, Bacillus, Clostridium, Pseudomonas* and *Serratia* (Law, 1979). They cause bitter, sour, fruity and unclean flavour defects in milk. These enzymes are thought to be essential in the degradation of triglycerides by lipases as they attack the phospholipid

component of the fat globule membrane (Sørhaug and Stepaniak, 1997; Cousin, 1982). Phospholipases produced by *B. cereus* cause what is known as the 'bitty cream' defect and causes floating clumps of fat in the milk (Robinson, 1990).

1.2.4.4 Heat Inactivation of Bacterial Extracellular Enzymes

The heat resistance of proteases produced by *Pseudomonas spp* is of particular concern to the dairy industry. As *Pseudomonas spp* often dominate the raw milk bacterial population, high populations of between 10^6 and 10^7 colony forming units per millilitre (CFU/ml) result in high levels of proteases being released into the milk. Once the milk has been pasteurised and stored under refrigeration for some time, heat resistant proteases produce bitter and off flavours in the milk (Moseley, 1980, Bishop and White, 1986). A number of studies have shown the heat resistance of these enzymes. Enzymes produced by *Ps. fluorescens* have shown D values of 304 min at 74°C (Cromie, 1992); 9 min at 120°C (Mayerhofer *et al.*, 1973); and 1min at 140°C (Cromie, 1992). Proteases are considered to be more than 4,000 times more resistant than *B. steorothermophilis* endospores when exposed to a temperature of 148°C (Bishop and White, 1986).

Lipases of many psychrotrophs are also heat resistant, having D values ranging from between 0.3 to 170 min at 72°C (Cousin, 1982) and 0.95 min at 140°C (Cromie, 1992) depending on the species. Lipases are, however, considered to be less resistant to high temperatures than proteases (Cromie, 1992).

Psychrotroph proteases, lipases and phospholipases are heat resistant but are not considered to be thermoenzymes because at high temperatures they are inactivated by covalent modifications that is, destruction of cystine cross-links and deamination of asparagine and glutamine residues and they are not active above 50-60°C. In comparison, thermoenzymes are heat stable and are highly active at temperatures of 60°C to 80°C. The stabilisation of thermoenzymes is due to additional salt bridges, hydrogen bonds and tighter Ca^{2+} binding sites. They are maximally packed, with shorter loops and expanded

hydrophobic cores (Sørhaug and Stepaniak, 1997). Psychrotroph enzymes share some of these structural features possessed by thermoenzymes and this equips them to resist high temperatures.

1.3 THE ORIGIN OF POST-PASTEURISATION CONTAMINATION

Pseudomonas spp dominate the raw milk and post-pasteurisation environment but are not thought to survive pasteurisation. Ralyea *et al.* (1998) found that 90% of *Pseudomonas spp* isolated were from the post-pasteurised environment and only 10% isolated were from raw milk. It has, therefore, been suggested that post-pasteurisation contamination by pseudomonads is due to an external contamination source, rather than surviving the pasteurisation process.

One external contamination source maybe by recontamination of bacteria from raw milk. A study by Reid (1997) used Multi Locus Enzyme Electrophoresis to identify one specific *Ps. fluorescens* cluster. This cluster was found throughout the factory, from the raw milk through to the packaged product. In a similar study by Ralyea *et al.* (1998) automated ribotyping was used to identify a *Ps. fluorescens* strain in pasteurised milk. This strain was found to be indistinguishable from one found in raw milk.

The alternative to raw milk recontamination of pasteurised milk would be contamination from an unknown external source. Research by Reid (1997) identified three strains of *Ps. fluorescens* which were endemic to the milk-processing factory. This means they were isolated from the post-pasteurised milk environment and were not found in the raw milk.

A possible source of contamination may be via the bottling room floor or drains as *Pseudomonad spp* are readily found in soil and water. Splashing from high-pressure hoses may wash bacteria into leaking gaskets, hairline or pinhole cracks in plates and on to processing equipment, hence introducing the contaminant into pasteurised milk (Bishop and White, 1986). Improper cleaning and sanitation of equipment can lead to biofilms

forming on stainless steel surfaces. In addition, airborne bacteria can also lead to contamination. A single *Ps. fluorescens* cell inoculated into a 2L bottle of milk, can multiply to 10^6 CFU/ml in 8 days at 7.2°C (Cousin, 1982). At this level of contamination organoleptic changes can be easily detected (Bishop and White, 1986).

1.4 SUMMER AND WINTER PSYCHROTROPHS

Psychrotrophic bacterial populations in raw milk vary considerably between seasons in both species type and population size. Summer populations are dominated by species of *Alcaligenes, Flavobacterium, Micrococcus* and *Pseudomonas*. Winter month populations are dominated by species of *Arthrobacter, Bacillus* and *Corynebacterium* (Andrey and Frazier, 1959).

A study by Uraz and Çitak (1998) found that pseudomonads were isolated at a higher frequency in the summer months than in the winter. Of the 48 pseudomonad colonies isolated from raw milk, 56% were isolated in the summer, 29% were isolated during the spring and only 15% were isolated during the winter. Populations of *Micrococcus* also showed seasonal changes with increases from 8.5% of the total bacterial population in the winter months to 33.4% in the summer months (Kikuchi and Matsui, 1976). *Bacillus spp* and *Corynebacterium* have shown population seasonal changes with these thermoduric psychrotrophs representing 27.3% of the total bacterial contamination in the winter milk compared to only 16.7% in the summer (Meer *et al.*, 1991).

There are two possible reasons for the changes in psychrotrophic populations of raw milk. One reason may be changes in animal diet. In the winter months cows are fed hay, grains or silage which contain higher levels of Gram-positive rods (Robinson, 1990) whereas, in the summer months they are pasture fed which leads to higher quantities of Gram-negative rods in the milk (Andrey and Frazier, 1959). Another reason may be changes in cleaning regimes and environmental conditions which may lead to population changes. During the summer, tanks are cleaned once daily and poor cleaning of these tanks may lead to

increased recontamination with Gram-negative rods. Higher temperatures may also lead to high populations of Gram-negative rods as they are able to grow faster and dominate the Gram-positive rods (Cousin, 1982).

The quantities of psychrotrophic bacteria found in the milk can also change considerably with up to a seven fold increase in bacterial populations in the summer months compared to those found in the winter (Andrey and Frazier, 1959, Kikuchi and Matsui, 1976).

1.4 AIMS

During the summer the milk-processing factory involved in this study receives numerous consumer complaints about their milk products. The greatest numbers of complaints concern bottled trim milk and involve milk spoiling four days before the consumer expiry date. Therefore, the main objective of this project was to assist in determining the nature and source of this contamination so that appropriate measures could be taken to extend the shelf-life.

Thus the objectives of this study were:-

- To monitor the types of bacteria present in bottled trim milk from immediately after bottling until the consumer expiry date and assess their proteolytic ability.

- To determine the possible origin of these bacteria.

- To establish seasonal changes in the type of bacteria found in raw milk, newly packaged milk and bottled milk at the consumer expiry date.

CHAPTER II

EXPERIMENTAL

2.1 SAMPLE COLLECTION

Milk samples used in this project were obtained from a Christchurch milk-processing factory and were taken at various times throughout the year from a number of sampling points on the milk-processing line. Other samples, such as environmental swabs, empty glass bottles and chlorinated factory water, were also used in this research. Hygiene and safety regulations imposed by the factory dictated that factory staff collected all samples. These were stored at 4°C for no longer than 6 hours before being transported to the laboratory.

Pre-existing milk sampling points are located on the processing line within the factory and consist of sampling elbows inserted into bends in the process line. These sampling points enable factory laboratory staff to monitor the microbial and chemical composition of the milk on a regular basis. A number of these points were sampled for this project and milk samples were collected into sterile 10ml tubes. The sites used included raw milk from a raw milk silo; newly pasteurised milk taken after pasteurisation but before the milk had reached the storage tanks and pasteurised milk from a pasteurised milk storage tank.

New milk sampling points were put in especially for this project including a point for pasteurised milk after leaving the storage tanks but before reaching the bottle filler and pasteurised milk taken from inside the filler rubbers. Pre-filler samples were taken by inserting a sampling elbow into the process line and removing samples in exactly the same way as the pre-existing sampling points. Filler rubber milk samples were obtained by inserting a syringe through the side of the filler rubber.

Commercially packaged milk samples were used and included glass bottles containing trim and homogenised milk, plastic bottles, sachets and cartons all containing trim milk. These samples were taken directly from the process line and removed after commercial capping or sealing.

Filler samples were taken from the glass bottle filler and placed in laboratory sterilised bottles. Milk was collected by removing a standard glass bottle from the process line and replacing it with a laboratory sterilised glass bottle. The size difference between the two types of bottle meant that the laboratory bottles could only be filled by manually pushing them up into the bottle filler. The laboratory bottles were sterilised by autoclaving them for 20 min at 121°C and 103.4 kPa.

Environmental swabs were taken with sterile cotton swabs which were soaked in sterile peptone water. There were five different swab sites: these included swabs from the filler head surround; a point adjacent to where the bottle rim contacts the filler rubber; the transfer wheel which diverts the flow of empty glass bottles near the bottle filler and swabs from the outside surface of the filler bowl. The fourth swab was taken from the factory worker steps to the filler and the final swab taken from a drain directly under the filler.

Non-milk samples assessed included chlorinated water samples from the CIP system and the bottle washer water supply, as well as empty glass bottles. The chlorinated water samples were taken from non-sterile taps, as there were no sterile sampling points present in this area of the factory, however, they were collected in sterilised bottles. Unfilled but capped glass bottles were also assessed for contamination.

2.2 BACTERIAL CONTAMINATION OF BOTTLED MILK

2.2.1 Changes in the Bacterial Populations of Bottled Milk

2.2.1.1 Isolation and Identification

To establish the changes in type and quantity of psychrotrophic bacteria found in bottled milk over a period of ten days, the following method was used. In late autumn, twelve 600ml bottles of trim milk were incubated for ten days at 7°C. Each day 100µl was removed from ten of these bottles and plated on to freshly made Luria Bertani (LB) medium (a non-selective agar) at the appropriate dilution. These plates were incubated for 2 days at 30°C and the microbial population determined in CFU/ml of milk. Two bottles remained sealed as controls to detect laboratory-based contamination. These were opened on day ten and the bacterial population in CFU/ml was established. Morphologically distinct colonies were isolated from all 12 plates. Morphological characteristics used included colony size, colour and surface texture, as well as the degree of transparency of colonies.

Primary identification of colonies was made according to Gram reaction (Hucker method, Doetsch, 1981) and the production of protease. A positive result for protease was shown by a clear halo around colonies grown on 1.6% milk medium (*Appendix I*, Figure 2.1). Gram-positive rods that produced proteases were identified using the BBL crystal system (as described in 2.2.1.2). Gram-negative rod colonies were tested for oxidase production. A positive result was shown by a blue colouration when exposed to oxidase reagent. Colonies testing positive for oxidase were identified by the API 20NE system (as described in 2.2.1.3).

2.2.1.2 The BBL Crystal Identification System

In order to classify Gram-positive rods isolated during this project the BBL Crystal system was used. This system combines tests for fermentation, oxidation, degradation and hydrolysis of various substrates as well as chromogen and fluorogen linked substrates

to detect enzymes produced by bacteria. A positive or negative result for each enzyme test translates into an allocated number and the combination of these numbers distinguishes the different bacterial types. The identification code is then entered into a computer database which matches it to the closest known bacterial species.

2.2.1.3 The API Identification System

The API system, like the BBL Crystal system, has different kit types depending on the type of bacteria being isolated. In this project the 20NE system was used, which is specific for Gram-negative, oxidase positive rods or bacteria belonging to the Enterobacteriaceae. The 20NE kit combines eight biochemical tests and thirteen assimilation tests; the biochemical tests producing colour changes when the appropriate enzyme is produced by the bacteria. Assimilation tests use minimal media containing specific substrates; a positive result is shown by colony growth. The result of each test translates into a number; the combination of these numbers distinguishes different bacterial types. The identification code is entered into a computer database which matches it to the closest known bacterial species.

Figure 2.1: *Protease Enzyme Activity on Milk Agar. The colonies shown on this milk agar plate are B. cereus (left), B. megaterium (top) and Ps. fluorescens (right). The clear halo around Ps. fluorescens and B. cereus indicates a positive result for proteases. The absence of a halo around B. megaterium indicates a negative result for proteases.*

2.2.1.4 Isolate Storage

Pure isolates were stored for a short-term period at 4°C on LB medium and for long-term at -80°C in 1.5ml LB broth tubes containing 15% glycerol.

2.2.2 Extent of Bottled Milk Bacterial Contamination

To determine the extent of bottled milk contaminated with psychrotrophic bacteria during late autumn, eighteen 600ml bottles of trim milk were incubated for ten days at 7°C. On day ten, 100µl from each bottle was plated on to two different media, LB medium and Goulds medium, a pseudomonad selective agar, in the appropriate dilutions. These plates were incubated at 30°C for one day on LB medium or two days on Goulds medium. Bacterial populations in CFU/ml for each type of medium were recorded and colonies of unique morphology were purified. Isolates were identified by Gram reaction and production of oxidase and protease. A select group of Gram-negative, oxidase and protease positive rods were identified further using the API 20NE system.

2.2.3 Changes in the Bacterial Population of Trim and Homogenised Milk

In order to validate the use of trim milk rather than homogenised milk to isolate psychrotrophic bacteria the following experiment was conducted. In mid-spring, two bottles of trim milk and two bottles of homogenised milk were incubated for eleven days at 7°C. Every two to three days 100µl of milk was plated on to LB medium and incubated at 25°C for two days and bacterial populations in CFU/ml were determined. Morphologically distinct colonies were isolated and identified by Gram reaction. Gram-positive rods were identified further by the BBL Crystal system.

2.3 TRACKING OF BACTERIAL CONTAMINATION IN THE MILK-PROCESSING FACTORY

2.3.1 Bacterial Population of Newly Pasteurised Milk over 28 Days

To determine whether psychrotrophic bacteria contaminate of milk immediately after pasteurisation the following method was used. In late spring, five samples of newly pasteurised, homogenised milk were incubated at 7°C for 28 days. Every two to five days 100µl of milk was removed from each tube and plated on to LB medium. These plates were incubated at 25°C for three to four days and bacterial populations in CFU/ml were determined. Morphologically distinct colonies were isolated and Gram reaction recorded. Colonies that were identified as Gram-positive rods were identified further by the BBL Crystal system.

2.3.2 Bacterial Population of Storage Tank Milk over 38 Days

To establish whether psychrotrophic bacterial contamination of milk occurred in the storage tanks the following samples were taken. In early summer, three samples of homogenised storage tank milk and three commercially filled bottles of homogenised milk were incubated at 7°C for 38 days and ten days respectively. Every two to four days, for the first ten days, 100µl was removed from each milk sample and plated on to LB medium. The three tubes of tank milk were also sampled on day 38. Plates were incubated at 25°C for 4-8 days and the bacterial population in CFU/ml for each sample determined. Morphologically distinct colonies were isolated and the Gram reaction was recorded. Colonies that were identified as Gram-negative rods were tested for oxidase production and then an API 20NE profile determined.

2.3.3 The Glass Bottle Filler as a Source of Bacterial Contamination

To discover the first point in which psychrotrophic bacterial contamination appeared in milk samples following the storage tanks the following method was used. In mid-autumn,

three samples of homogenised tank milk, pre-filler milk and filler rubber milk as well as three bottles of homogenised milk were incubated for ten days at 7°C. On days seven and ten 100µl from each sample were removed and plated on to LB medium. These plates were incubated for between one to four days at 25°C and the bacterial populations in CFU/ml were determined. Morphologically distinct colonies were isolated and Gram reaction recorded. Gram-negative rods were tested for production of oxidase and an API 20NE profile determined. A small group of Gram-positive rod colonies were also identified further by the BBL Crystal system.

2.3.3.1 Wear on Filler Rubber Surface

To determine the degree of wear on the inner surface of filler rubbers the following method was used. Two filler rubbers were obtained from the milk-processing factory; one was new and unused, whereas the other one was used and had been removed during routine replacement. These two filler rubbers were cut in half to examine their inner surface and a surface section was cut out for examination by scanning electron microscope.

2.3.4 Possible Origin of External Bacterial Contamination

2.3.4.1 Chlorinated Water

To eliminated the factory water as a possible source of psychrotrophic bacterial contamination the following method was used. In late winter and mid-spring, four 10ml samples of CIP tank water and bottle line chlorinated water were obtained. One millilitre of each sample was spread over ten LB plates and incubated at 25°C for five days. The bacterial population in CFU/ml was determined and all colonies isolated were identified according to their Gram reaction. A single oxidase positive colony of Gram-negative rods was identified further using an API 20NE kit.

2.3.4.2 Recycled Glass Bottles

To eliminate recycled glass bottles as sources of psychrotrophic bacterial contamination two methods were used. Firstly, in late spring, two empty bottles were obtained. These

had been commercially capped, but not filled. Three millilitres of peptone water was aseptically injected into each bottle, the solution was swirled around and then 1ml was plated on to each of ten LB plates. The plates were incubated at 25°C for five days and bacterial populations in CFU/ml determined. In early summer, three commercially filled bottles of homogenised milk and three bottles of the same homogenised milk were collected in laboratory sterilised bottles. These six bottles were incubated at 7°C for 10 days and every two to four days, 100µl of milk was removed from each bottle and plated on to LB medium. Plates were incubated at 25°C for six to eight days and bacterial populations determined. Morphologically distinct colonies were isolated and Gram reaction recorded. Colonies that were identified as Gram-negative rods were also tested for production of oxidase and an API 20NE profile obtained.

2.3.4.3 Environmental Swabs

To identify surfaces within the glass bottle filling room which might harbour psychrotrophic bacteria, environmental swabs using sterile cotton swabs were taken. Duplicate swabs were taken during early autumn from each site described in 2.1. Swabs were spread directly on to LB medium in a twisting motion and the plates were incubated at 25°C for two days. The bacterial population in CFU was determined and morphologically distinct colonies were isolated and tested for Gram reaction. Colonies identified as Gram-negative rods were tested for oxidase and API 20NE profile obtained.

2.4 SEASONAL VARIATION IN BACTERIAL CONTAMINATION OF MILK

In order to establish changes in the type and quantity of psychrotrophic bacteria contamination found in milk through out the year the following method was used. In late summer and early winter, samples of raw milk, newly pasteurised trim milk, storage tank trim milk and four different trim milk packaged products were obtained from the milk-processing factory. Duplicate samples were taken for raw milk, newly pasteurised milk and tank milk; a single sample was taken from each of the packaged products. Samples

were plated on to LB medium and Milk Plate Count Agar (MPCA). Plates were incubated for seven days at 10°C and then three days at 25°C. Bacterial populations were determined in CFU/ml and morphologically distinct colonies isolated. Colonies identified as Gram-negative rods were tested for oxidase. A single colony of Gram-negative, oxidase positive rods was identified further by the API 20NE system.

CHAPTER III

RESULTS

3.1 BACTERIAL CONTAMINATION OF BOTTLED MILK

3.1.1 Changes in the Bacterial Population of Bottled Milk

In order to determine the changes in the bacterial population of bottled milk, the dominant bacterial populations present immediately after bottling were established. These populations were compared to those found ten days later at the consumer expiry date.

The bacterial population followed a standard bacterial growth curve, as shown in Figure 3.1. The initial bacterial population was recorded at $1.9x10^2$ CFU/ml and over the next three days the population entered a lag phase. From day three to day six an exponential phase of growth ensued with populations reaching $3.2x10^6$ CFU/ml. In the remaining four days, the bacterial population entered a long stationary phase and remained static.

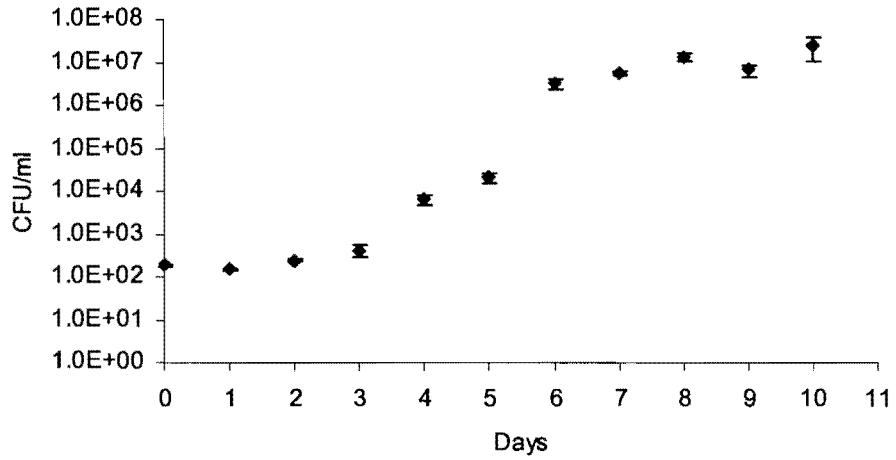

Figure 3.1: *The Bacterial Population of Bottled Trim Milk over ten days. Bottles were incubated at 7°C and samples plated on to LB medium. The error bars were calculated from the standard deviation of the mean.*

Control bottles were used to detect laboratory-based contamination from the sampling methods used. Milk samples from these bottles produced bacterial populations of $1.4x10^8$ and $1.0x10^7$ CFU/ml. The control bacterial populations were compared to populations found in the remaining 10 bottles of this study. These populations ranged between $2.5x10^6$ to $1.5x10^8$CFU/ml and thus show that laboratory-based contamination was minimal.

The types of bacteria dominating the bacterial populations of bottled milk are shown in Figure 3.2. In the first two days of the lag phase 80% of the ten colonies isolated were identified as Gram-positive rods. Of these, however, only one colony showed protease activity and this was identified as *B. licheniformis* (Table 3.1). Colonies isolated at the beginning of the exponential phase were found to be a mixture of bacterial types. The Gram-positive rods identified were generally not protease producers; however, there were exceptions, the most notable being a colony identified as *B. subtilis*. The Gram-negative rods isolated from this period were identified as *Ps. putida* and *Ps. fluorescens* and only some tested positive for protease. Of the colonies isolated, at the end of the exponential phase and in the stationary phase, 71% or 22 colonies were identified as Gram-negative rods and of these eighteen percent produced protease.

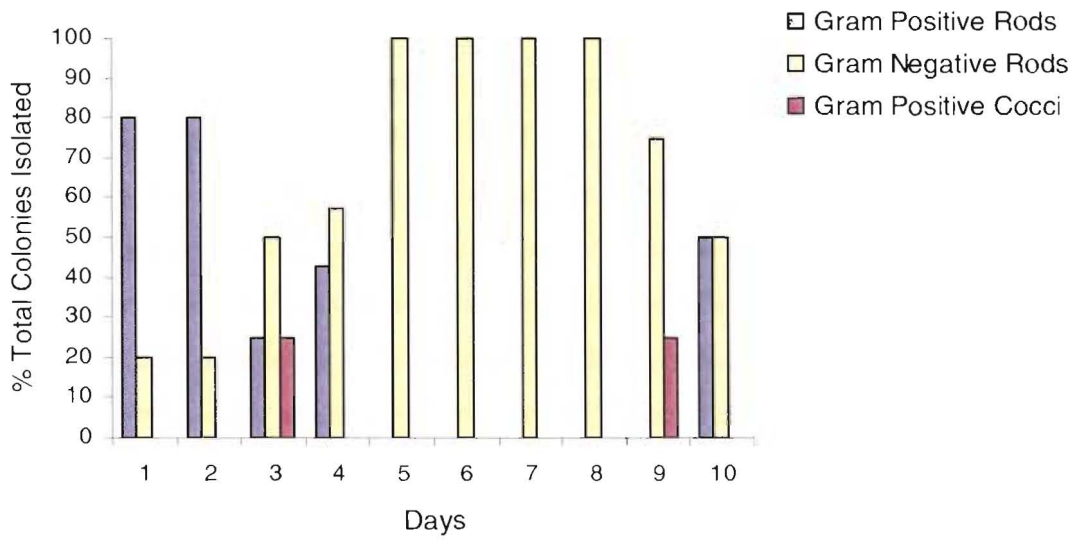

Figure 3.2: *Changes in the Type of Bacteria Identified in Bottled Trim Milk, over ten days.*

Table 3.1: *Identification of Bacteria Isolated from Bottled Milk over Ten Days.*

Day Isolated	Colony #	Oxidase	Gram Stain	Protease	Identification	ID Type	Code	Biotype
1	h	Negative	Positive rod	Positive	*B. licheniformis*	BBL	2334000773	--
3	a	Positive	Negative rod	Positive	*Ps. putida*	API 20NE	4143455	6p
3	b	Positive	Negative rod	Positive	*Ps. putida*	API 20NE	O343455	7p
3	g	Positive	Negative rod	Negative	*Ps. fluorescens*	API 20NE	O346455	6f
4	c	Negative	Positive rod	Positive	*B. subtilis*	BBL	2710453643	--
6	d	Positive	Negative rod	Negative	*Ps. fluorescens*	API 20NE	O346455	6f
6	e	Positive	Negative rod	Positive	*Ps. putida*	API 20NE	O143455	5p
6	f	Positive	Negative rod	Positive	*Ps. putida*	API 20NE	O143445	8p
8	d	Positive	Negative rod	Negative	*Ps. putida*	API 20NE	O141455	4p
8	e	Positive	Negative rod	Negative	*Ps. putida*	API 20NE	O143445	8p
9	e	Positive	Negative rod	Negative	*Ps. putida*	API 20NE	O343455	7p
10	f	Positive	Negative rod	Positive	*Ps. fluorescens*	API 20NE	O153455	7f

3.1.2 Extent of Bottled milk Bacterial Contamination

To establish the extent of bottled milk bacterial contamination, the proportion of the eighteen bottles sampled which contained high bacterial populations on their consumer expiry date was determined. Dairy regulations (Ministry of Health, 2002) consider that milk which contains $5.0x10^5$ CFU/ml or more, has a bacterial population which is too high for retail sale.

The bacterial population of the bottles was found to range between $1.8x10^6$ and $9.8x10^7$ CFU/ml on LB medium (*Appendix II*), which meant that all eighteen bottles sampled exceeded the threshold and would be considered unfit for sale. Bacterial populations isolated on Goulds medium ranged between $6.0x10^4$ and $5.0x10^7$ CFU/ml. Eleven of the eighteen bottles exceeded the $5.0x10^5$ CFU/ml threshold and hence were spoilt by *Pseudomonas spp.* To support this finding, the bacterial populations isolated on both types of media were identified. Of the 106 colonies isolated, 73% were identified as Gram-negative rods, 22% as Gram-positive rods and 5% as Gram-positive cocci. The Gram-negative rods isolated were assessed for their production of oxidase and protease; 81% tested positive for oxidase and of these 55% tested positive for protease. Thirteen oxidase and protease positive colonies were identified further using the API 20NE system (Table 3.2); ten colonies were identified as *Ps. putida* and three as *Ps. fluorescens.*

Table 3.2: *Identification of Bacteria Isolated from of Bottled Milk at Expiry Date.*

Date Isolated	Colony #	Medium	Protease	Oxidase	Gram stain	API Identification	API code	Biotype
2 May	1b	LB	Positive	Positive	Negative rod	*Ps. putida*	4353455	1p
	2a	LB	Positive	Positive	Negative rod	*Ps. putida*	4353455	1p
3 May	1a	LB	Positive	Positive	Negative rod	*Ps. putida*	4141455	2p
	1c	G	Positive	Positive	Negative rod	*Ps. putida*	O143455	3p
	4e	G	Positive	Positive	Negative rod	*Ps. fluorescens*	O157555	1f
4 May	1c	LB	Positive	Positive	Negative rod	*Ps. fluorescens*	1353455	2f
	1a	G	Positive	Positive	Negative rod	*Ps. putida*	O141455	4p
	4d	G	Positive	Positive	Negative rod	*Ps. putida*	O143455	3p
	4e	G	Positive	Positive	Negative rod	*Ps. fluorescens*	O156575	3f
5 May	1a	LB	Positive	Positive	Negative rod	*Ps. putida*	4141455	2p
	1c	LB	Positive	Positive	Negative rod	*Ps. putida*	4343455	5p
	1c	G	Positive	Positive	Negative rod	*Ps. putida*	4141455	2p
	2b	G	Positive	Positive	Negative rod	*Ps. putida*	4353455	1p

3.1.3 Changes in the Bacterial Populations of Trim and Homogenised Milk

A comparison of the level and type of bacteria which contaminate trim and homogenised bottled milk was made in order to determine any predisposition for contamination with respect to milk type.

Bacterial populations of the two types of milk are shown in Figure 3.3. Both milk populations followed a standard bacterial growth curve. The lag phase began with bacterial populations of $1.5x10^2$ and $9.0x10^1$ CFU/ml for trim and homogenised milk respectively and persisted through to day five. The exponential and the stationary phases appeared very short, enduring only two days for each. The difference between the milk types was slight: homogenised milk bacterial populations increased more in the exponential phase resulting in a bacterial population of $5.0x10^5$ CFU/ml greater than the trim milk bacterial population.

The bacterial types that dominated trim and homogenised milk over the eleven days differed only slightly, as shown in Figures 3.4a and 3.4b. For the first three days of sampling Gram-positive cocci dominated both populations. From day five to day eleven, however, Gram-positive rods dominated trim milk entirely. Homogenised milk was also

dominated by Gram-positive rods but only at a level of 50% of the total bacterial population. These bacteria were identified as *Bs. Cereus.*

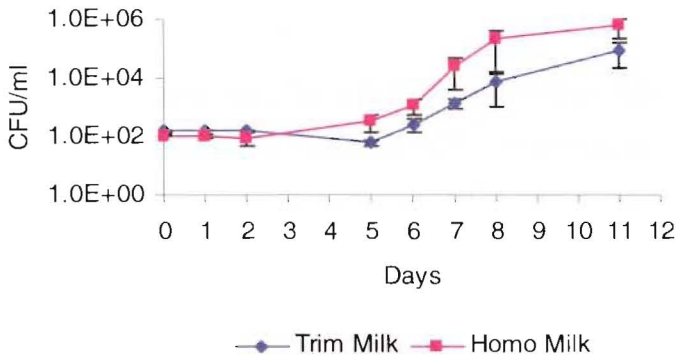

Figure 3.3: *The Bacterial Population of Bottled Trim and Homogenised (Homo) Milk.*

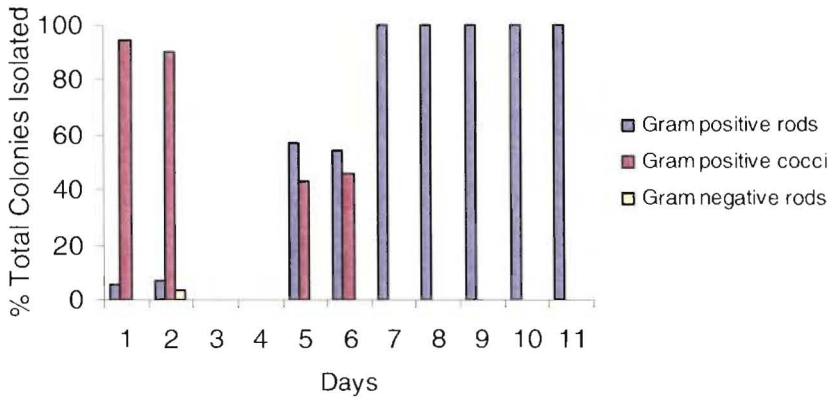

Figure 3.4a: *Changes in the Type of Bacteria Identified in Trim Milk.*

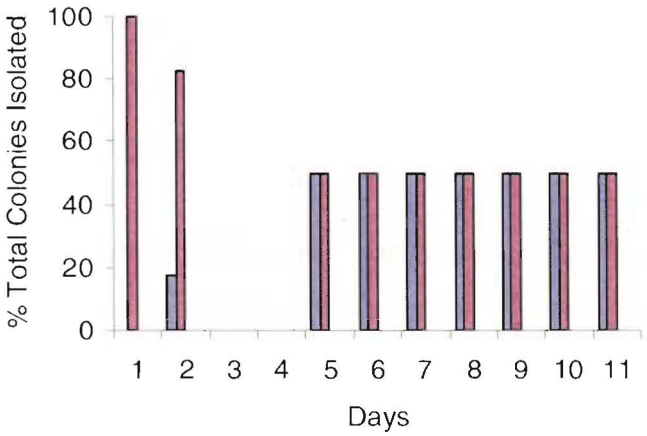

Figure 3.4b: *Changes in the Type of Bacteria Identified in Homogenised Milk*

3.2 TRACKING OF BACTERIAL CONTAMINATION IN THE MILK-PROCESSING FACTORY

3.2.1 Bacterial Population of Newly Pasteurised Milk over 28 Days

Milk samples were taken directly after pasteurisation but before they entered the pasteurised milk storage tanks so that the level of bacteria surviving pasteurization could be determined.

The bacterial population initially was $1.1x10^2$ CFU/ml, as shown in Figure 3.5. The population remained static for the next thirteen days and then gradually increased to $3.3x10^5$ CFU/ml after day twenty eight.

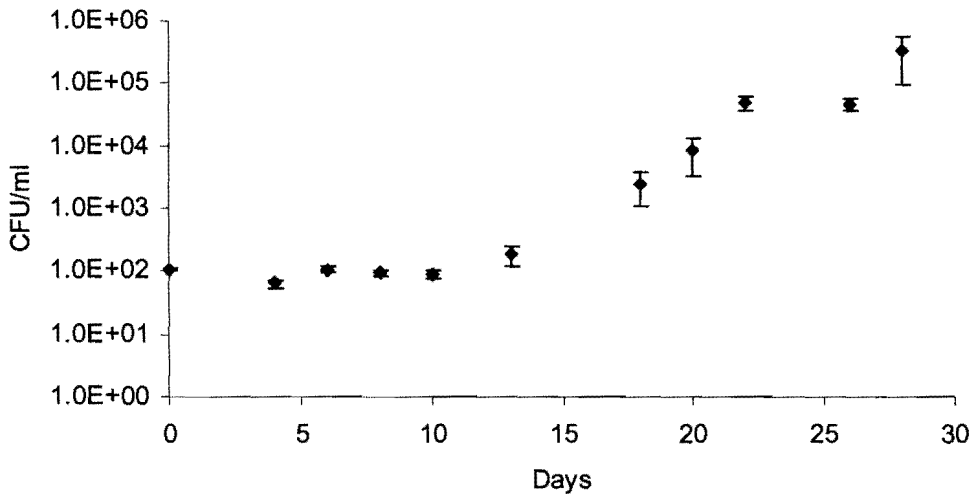

Figure 3.5: *The Bacterial Population of Newly Pasteurised over twenty eight days. Bottles were incubated at 7°C and samples plated on to LB medium. The error bars were calculated from the standard deviation of the mean.*

The dominant type of bacteria in newly pasteurised milk changed dramatically over twenty eight days, as shown in Figure 3.6. Initially through to day ten Gram-positive cocci dominated the population, whereas from day eighteen to day twenty eight Gram-

positive rods dominated. These colonies were identified as *B. cereus, B. circulans* and a *Corynebacterium sp.*

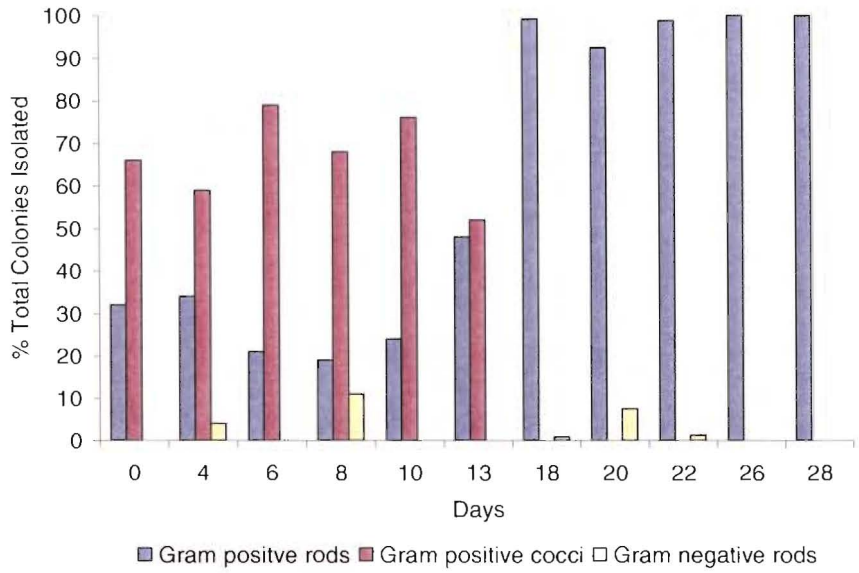

Figure 3.6: *Changes in the Type of Bacteria Identified in Newly Pasteurised Milk over twenty eight days.*

3.2.2 Bacterial Population of Storage Tank Milk over 38 Days

Storage tank milk populations were determined to establish whether contamination began within or prior to entry into the milk storage tanks. The populations present in tank milk were compared to bacterial populations found in bottled milk from the same process batch.

Tank milk bacterial populations initially were 2.7×10^2 CFU/ml, as shown in Figure 3.7. For the next four days the populations remained unchanged. By day eight, however, populations had increased and from here there was a steady increase until day thirty eight when the population reached 1.4×10^6 CFU/ml. The bottled milk bacterial population increased in a very different pattern; initially populations were similar in size to the tank milk, but by day ten the bacterial population had increased rapidly, resulting in a population of 1.2×10^7 CFU/ml.

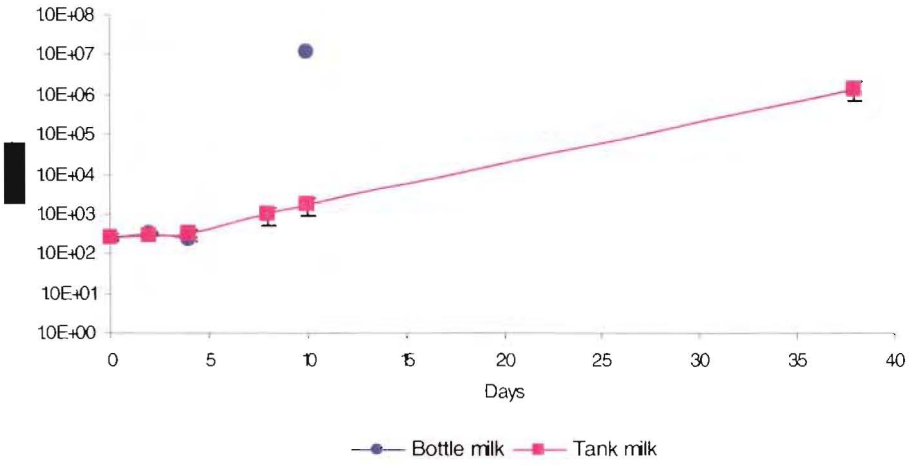

Figure 3.7: *The Bacterial Populations of Tank and Bottled Milk over 38 days. Bottles were incubated at 7°C and samples plated on to LB medium. The error bars were calculated from the standard deviation of the mean.*

The type of bacteria which dominated tank milk are shown in Figure 3.8a. There were three changes in dominant bacterial types. Initially, Gram-positive cocci predominated but by day ten Gram-positive rods had outgrown them. Finally, when samples were taken on day thirty eight, Gram-negative rods dominated and were identified as *Sphingomonas paucimobilis*.

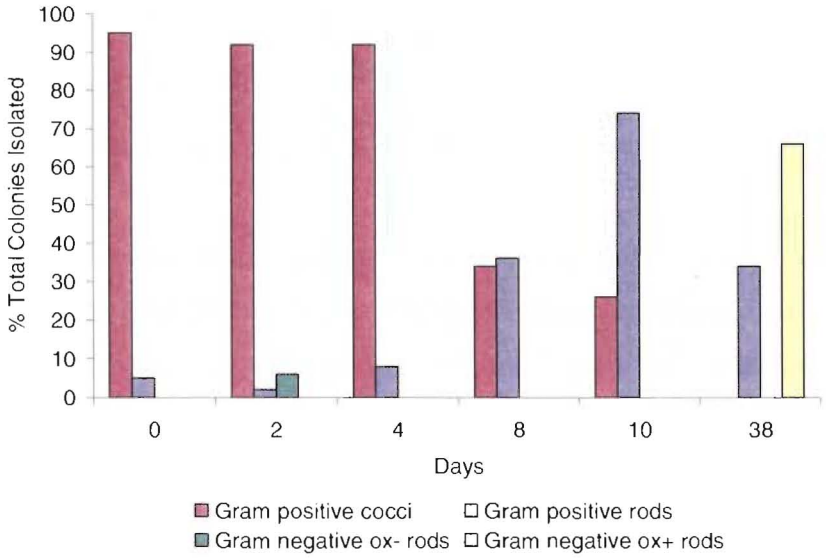

Figure 3.8a: *Changes in the Type of Bacteria Identified in Tank Milk over 38 Days*

Bacterial types identified in bottled milk followed a similar trend initially; Gram-positive cocci dominated as shown in Figure 8b. By day ten, however, Gram-negative rods dominated, which were identified as *Ps. fluorescens* and *Ps. putida* (shown in Table 3.3).

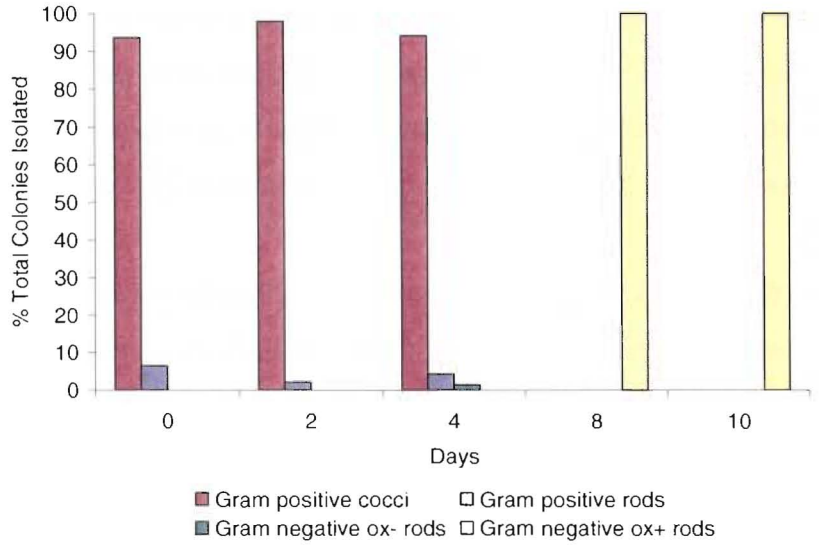

Figure 3.8b: *Changes in the Type of Bacteria Identified in Bottled Milk over Ten Days.*

Table 3.3: *Identification of Bacteria Isolated from Bottled Milk in Tank Milk Study.*

Day Isolated	Colony #	Medium	Oxidase	Gram Stain	API Identification	API Code	Biotype
8	C	LB	Positive	Negative rods	*Ps. fluorescens*	O357555	4f
8	D	LB	Positive	Negative rods	*Ps. fluorescens*	O157575	5f
8	E	LB	Positive	Negative rods	*Ps. fluorescens*	O357555	4f
8	F	LB	Positive	Negative rods	*Ps. putida*	O143455	3p

3.2.3 The Glass Bottle Filler as a Source of Bacterial Contamination

The dairy industry has historically attributed bacterial contamination of milk to the bottle filler, specifically the filler rubbers (Chadwich-Hayes and Boor, 2001). This hypothesis was tested by comparing the bacterial population of milk samples taken from: the storage tanks; the pre-filler (a point after the storage tanks but before the bottle filler bowl); from inside the filler rubbers themselves and from commercially filled bottles of homogenised milk.

The bacterial populations of milk samples taken from early in the process line such as from the tank and pre-filler were very different from those taken from the filler rubber

and finished product. The tank and pre-filler samples remained low over the ten days with populations of $1.1x10^4$ and $4.2x10^3$ CFU/ml respectively. The samples taken from the filler rubbers and bottles, however, reached populations of $4.7x10^4$ and $3.5x10^5$ CFU/ml after only seven days (*Appendix II*).

The filler rubber and bottle samples differed significantly between samples taken from the same site. The filler rubber samples taken on day seven varied between 30 and $1.4x10^5$ CFU/ml and bottled samples varied between 300 and $1.0x10^6$ CFU/ml. This meant that the average bacterial populations obtained for these sites was unreliable.

The types of bacteria isolated also varied between sites (*Appendix II*). Colonies isolated from tank milk were identified as entirely Gram-positive rods, whereas isolates from the pre-filler and the filler rubber samples were a mixture of Gram-positive cocci and Gram-positive rods. The most notable Gram-positive rod isolated from the pre-filler and was identified as *B. cereus*. There were three different types of bacteria isolated from bottle samples: Gram-positive cocci, Gram-positive rods and a Gram-negative ox+ rod. This last bacterium was further identified as *Ps. fluorescens*.

3.2.3.1 Wear on Filler Rubber Surfaces.

The filler rubbers were examined by obtaining a used rubber and comparing the inner surface texture to a new, unused filler rubber. Figures 3.9 a and b show cross sections of the new and used filler rubbers. Figure 3.9c shows a comparison between the two filler rubbers when they are flexed, which would normally occur during the filling process. Figure 3.10a shows the surface of the new filler rubber under the SEM and Figure 3.10b shows the surface of the old filler rubber. The used filler rubber shows general wear over the entire inner surface; cracks appear on bends and when it is flexed. The SEM also shows deep cracks and the highly porous nature of the rubber.

Figure 3.9 a: *A cross section of a new filler rubber.*

Figure 3.9 b: *A cross section of a used filler rubber.*

Figure 3.9 c: *A comparison between a used (left) and new (right) filler rubber when flexed.*

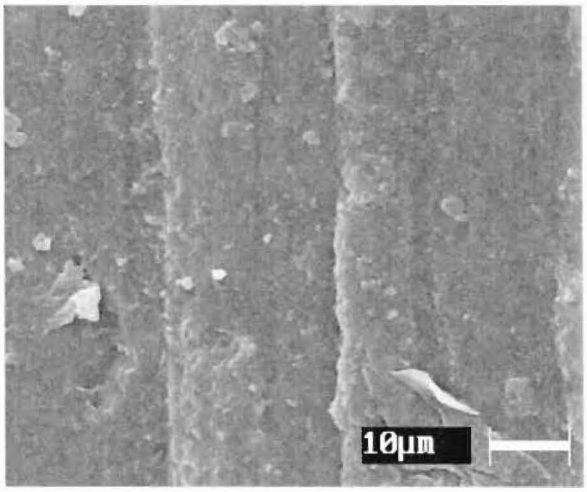

Figure 3.10 a: *SEM image of the inner surface of a new filler rubber.*

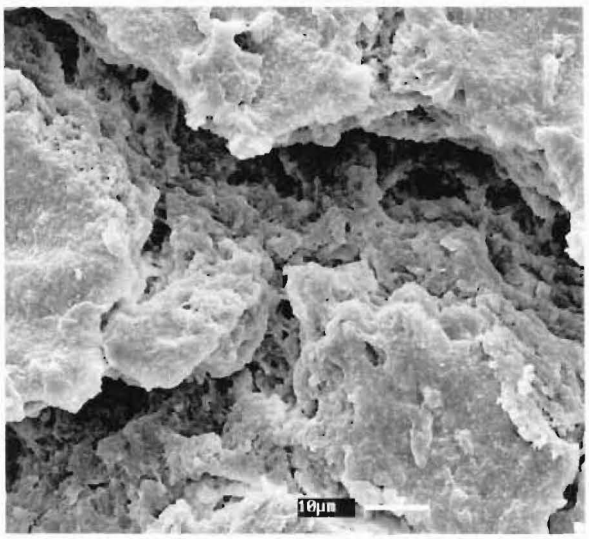

Figure 3.10 b: *SEM image of the inner surface of a used filler rubber.*

3.2.4 Possible Origin of External Bacterial Contamination

3.2.4.1 Chlorinated Water

Chlorinated water is used in the final rinse of the CIP system and the final rinse of the recycled glass bottles, it is likely then that small amounts of this water contaminate the milk at the beginning of the milk processing cycle every day and in each recycled bottle used.

Direct plating of water samples on to LB medium produced only six colonies for all four replications of the method. The CIP system samples produced one colony per ten plates or 1 CFU/ml and the bottle line samples produced one colony per twenty plates or 0.5 CFU/ml. The six colonies isolated were identified as four Gram-positive cocci, one Gram-positive rod and one Gram-negative rod. This Gram-negative rod was identified further as either *Sphingomonas spiritvorum* or *Chryseobacterium meningosepticum.*

3.2.4.2 Recycled Glass Bottles

The glass bottles used by this milk processing factory are recycled and undergo a rigorous cleaning process. Despite this, bacterial contamination may still be present on the glass surface.

No bacterial contamination was found in the factory-capped empty bottles sampled.

The laboratory sterilised bottle method, however, produced a range of bacterial types with numbers (Figure 3.11) varying only slightly when compared to a standard recycled bottle. Initial bacterial populations for both bottles were very similar with $2.3x10^2$ and $2.4x10^2$ CFU/ml for the standard and sterilised bottles respectively. For the next two days the bacterial population for both bottles remained stationary but by day ten, populations of both samples had increased exponentially. The bacterial population of the standard bottle on day ten was higher than the sterilised bottle by $9.2x10^6$ CFU/ml. When the error bars (the standard error of the mean value) were taken into account, however, the difference in the bacterial populations between the two types of collection bottles was minimal.

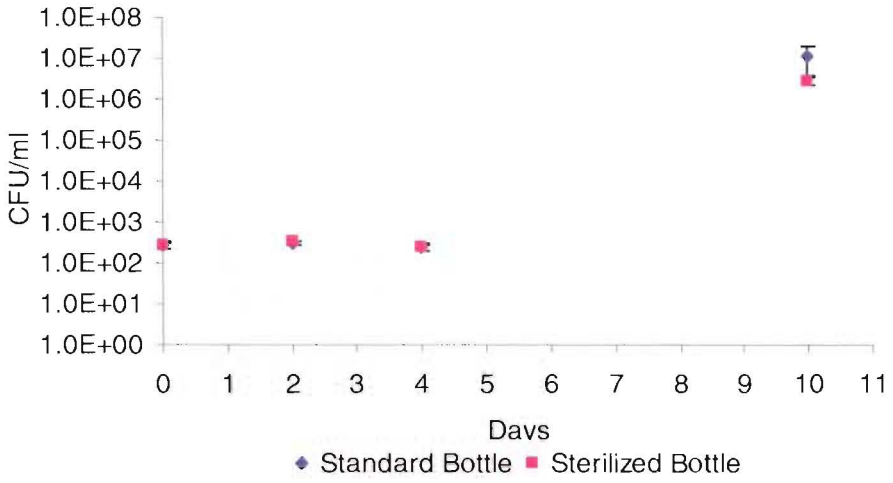

Figure 3.11: *The Bacterial Populations of Homogenized Milk Collected in Laboratory Sterilized Bottles and Standard recycled bottles. The error bars were calculated from the standard deviation of the mean.*

Bacterial types from both bottles followed similar trends, as shown in Figures 3.12a and b. Gram-positive cocci dominated bacterial populations entirely for the first three days: by day eight and day ten, however, Gram-negative ox+ rods had taken over both bottles. These bacteria were further identified as *Ps. fluorescens* and *Ps. putida* as shown in Table 3.4.

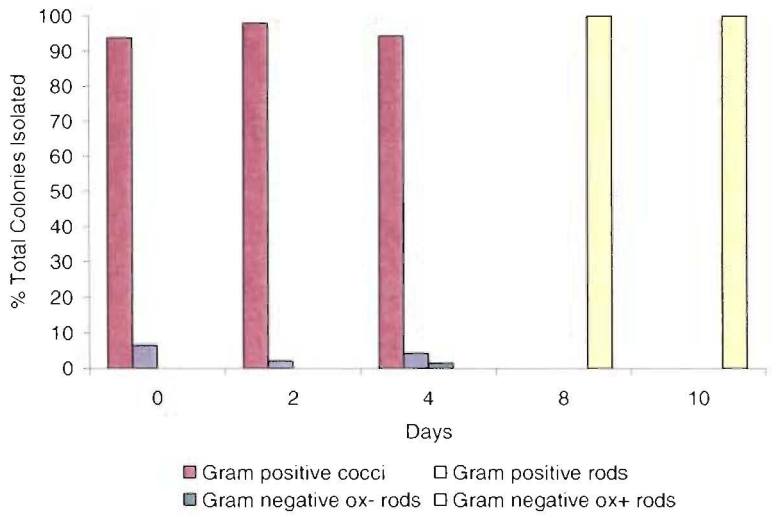

Figure 3.12a: *Changes in the Type of Bacteria Identified in Milk collected in Standard Bottles.*

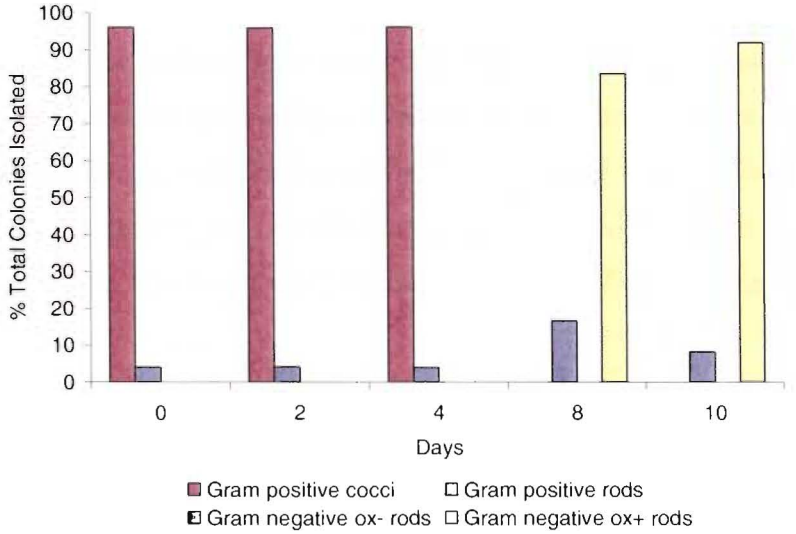

Figure 3.12b: *Changes in the Type of Bacteria Identified in Milk Collected into Sterilised Bottles.*

Table 3.4: Identification of Bacteria Isolated from Sterilised and Standard Bottled Milk.

Day isolated	Bottle Type	Colony #	Media	Oxidase	Gram Stain	API Identification	API Code	Biotype
8	Standard	C	LB	Positive	Negative rods	*Ps. fluorescens*	O357555	4f
8	Standard	D	LB	Positive	Negative rods	*Ps. fluorescens*	O157575	5f
8	Standard	E	LB	Positive	Negative rods	*Ps. fluorescens*	O357555	4f
8	Standard	F	LB	Positive	Negative rods	*Ps. putida*	O143455	3p
8	Sterilised	G	LB	Positive	Negative rods	*Ps. fluorescens*	O157555	1f
8	Sterilised	H	LB	Positive	Negative rods	*Ps. fluorescens*	O357555	4f
8	Sterilised	I	LB	Positive	Negative rods	*Ps. fluorescens*	O157555	1f

3.2.4.3 Environmental Swabs

Environmental swabs were taken from around the glass bottle filler room. These were designed to detect possible bacterial aerosols and contamination carried in by the factory staff. Samples included were from the filler head surround, the filler bowl, the factory steps to the filler and a drain under the filler, as well as a transfer wheel near the filler.

The filler bowl and factory worker steps produced the highest bacterial growth with populations of 1.5×10^3 and 3.1×10^2 CFU. The filler head and drain produced a much

lower population with 10 and 39 CFU respectively and the transfer wheel showed very little growth with only one colony isolated (*Appendix II*).

The types of bacteria isolated from the environmental swabs are shown in Table 3.5. The dominant types from all the surfaces swabbed were Gram-negative ox+ rods. A small group of these bacteria were identified further and were found to belong to a range of species including *Aeromonas, Burkholderia* and *Chryseobacterium spp.* Only one *Ps. fluorescens* (biotype 1f) colony was isolated and this was found on the factory stairs.

Table 3.5: *Bacterial Types Isolated from Environmental Swabs*

Swab Samples	Gram positive rods	Gram positive cocci	Gram negative ox+ rods	Gram negative ox- rods	Colony Total
Filler head	2		2	1	5
Filler bowl	4	2	7	6	18
Steps	4	2	7	2	15
Drain	6	1	6	2	15
Transfer wheel		1			1

3.3 SEASONAL VARIATION IN BACTERIAL CONTAMINATION OF MILK

3.3.1 Initial Bacterial Populations of Raw and Pasteurised Milk

Seasonal differences in the initial populations of bacteria contaminating milk were determined by sampling various sites along the milk process line during the summer and winter. The milk samples taken included raw milk, newly pasteurised milk, tank milk and milk from four different types of packaged products. Two different types of media were used, MPCA which is used by dairy laboratories and LB medium which is an alternative non-selective medium. The reason for using these two types of media was to isolate a large range of bacteria which may not be identified on MPCA, effectively justifying the use of MPCA to isolate bacterial contamination, as well as to vindicate the use of LB medium in this research.

The difference between bacterial populations found in winter and the summer milk are shown in Figures 3.13a and b.

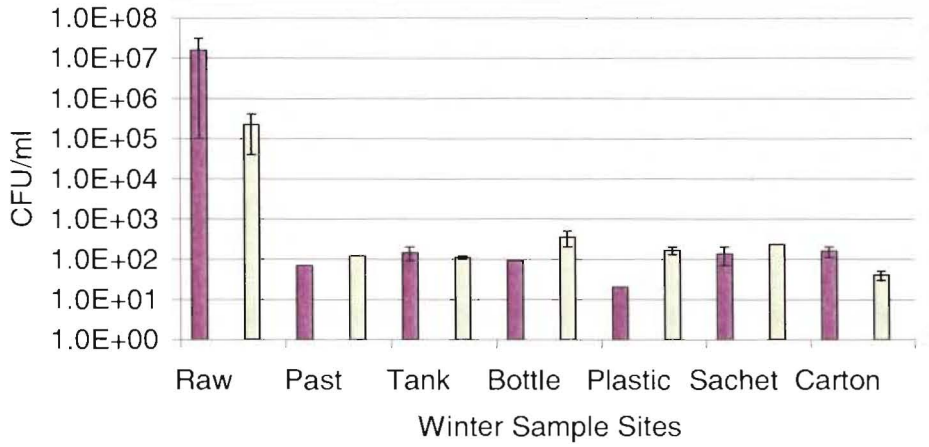

Figure 3.13 a: *The Bacterial Population of Winter Milk. The error bars were calculated from the standard deviation of the mean.*

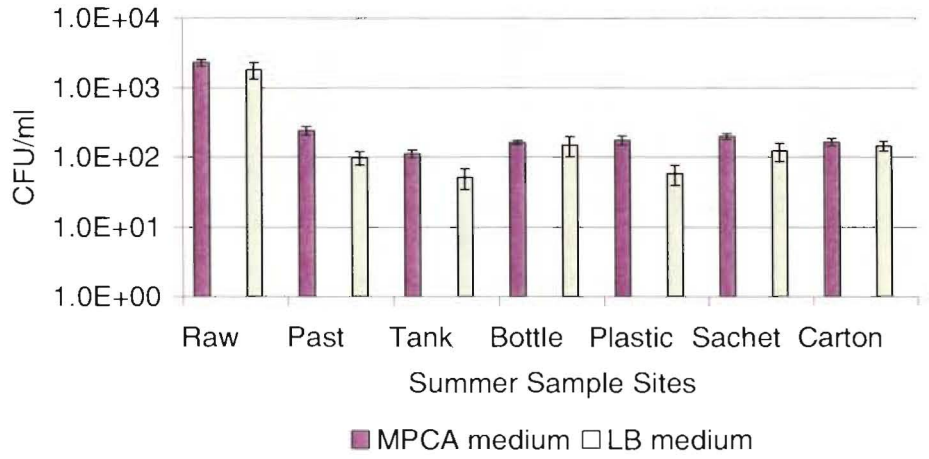

Figure 3.13 b: *The Bacterial Population of Summer Milk*

The bacterial populations of most samples were very similar between the two seasons; the only exception being the raw milk samples. The bacterial populations found in the raw milk ranged between $1.8x10^3$ and $2.3x10^3$ CFU/ml in the summer for LB medium and MPCA medium respectively, whereas in the winter bacterial populations ranged between $2.2x10^5$ and $1.6x10^7$ CFU/ml for LB medium and MPCA medium respectively.

The sizes of bacterial populations isolated on LB medium compared to MPCA medium were very similar. Of the fourteen milk samples taken throughout the year, nine samples

isolated on MPCA medium were higher in population than those isolated on LB medium. The largest discrepancy was in raw milk samples isolated during the winter. These samples showed differences in the average bacterial population of $1.4x10^6$ CFU/ml, but when standard error was taken into account, the population differences between the two media were not significant.

The types of bacteria isolated from raw milk in the two seasons differed, as shown in Figures 3.14 a and b.

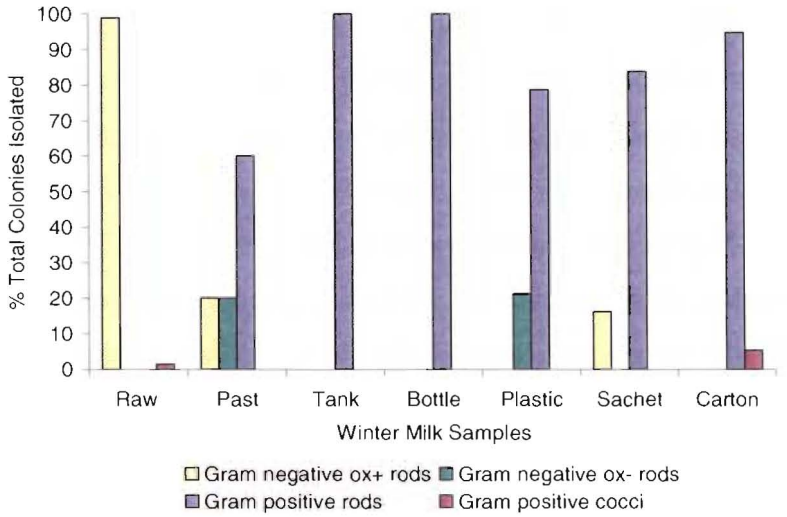

Figure 3.14a: *Changes in the Type of Bacteria Identified in Winter Milk.*

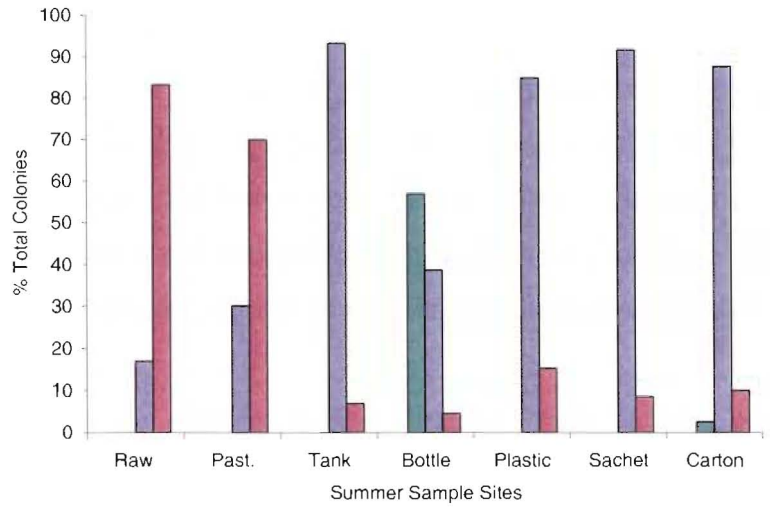

Figure 3.14 b: *Changes in the Type of Bacteria Identified in Summer Milk*

Gram-positive cocci dominated the summer raw milk bacterial populations, whereas the winter milk was dominated entirely by Gram-negative ox+ rods; one such colony was identified as *Ps. putida*, biotype 6p (*Appendix 2*). Newly pasteurised milk, like raw milk, differed in bacterial population type between the seasons. Gram-positive cocci dominated the summer populations, whereas in the winter, Gram-positive rods dominated. The tank milk and the four different types of packaged products had bacterial populations that were very similar between the seasons with Gram-positive rods dominating both the summer and winter milk. The only exception was bottled milk which was dominated by Gram-negative ox- rods in the summer.

There was very little difference in the types of bacteria isolated on the two different media; however, there were slight differences in the proportion of bacterial types isolated from some sample sites. Bacterial populations isolated in the summer from newly pasteurised milk were dominated entirely by Gram-positive cocci on LB medium, whereas on MPCA medium, populations were dominated by 80% Gram-positive rods and only 20% Gram-positive cocci. Likewise, in the tank milk and the packaged products Gram-positive rods constituted 73% of the population when isolated on LB medium, but on MPCA medium they only constituted 52%, with the remaining populations identified as Gram-positive cocci.

3.3.2 Bacterial Populations of Bottled Milk on Expiry Date

Seasonal variations in the bacterial populations contaminating bottled milk on the expiry date were established by comparing the bacterial populations found at various times throughout the year. There were four different times during this research when bacterial populations of trim and homogenised bottled milk were established: in late autumn, mid-spring and early summer, as well as mid-autumn the following year.

Seventeen out of the eighteen bottles obtained in late autumn, early summer and mid autumn the following year were found to contain bacterial populations that exceeded the $5.0x10^5$ CFU/ml Dairy regulations (Ministry of Health, 2002) threshold. These bottles

were all found to contain high levels of Gram-negative ox+ rods identified as *Ps putida* and *Ps fluorescens*. In mid-spring, however, one in six bottles sampled was found to contain bacterial populations exceeding $5.0x10^5$ CFU/ml; these bottles contained both Gram-positive rods and Gram-positive cocci.

CHAPTER IV

DISCUSSION

Psychrotrophic bacterial contamination of milk is a large concern to the dairy industry on a global level and New Zealand is no exception. The psychrotrophic bacteria of most concern are of two types: Gram-negative, oxidase positive rods such as *Pseudomonas spp* and Gram-positive, spore-forming rods such as *Bacillus spp* (Cousin, 1982). Pseudomonads are thought to contaminate pasteurised milk during the filling process (Dogan and Boor, 2003) and are considered solely post-pasteurisation contaminants (Cousin, 1982), whereas *Bacillus spp* have been shown to produce heat resistant spores and contaminate pasteurised milk by surviving pasteurisation (Meer *et al.*, 1991). In a Christchurch milk-processing factory, growth of psychrotrophic bacteria in milk has caused numerous consumer complaints. These complaints concern the increase in unfavourable organoleptic traits four days prior to the consumer expiry date. The main objective of this research was to establish the type, origin and seasonal variations of psychrotrophic bacteria contaminating milk from this particular milk-processing factory so that appropriate control measures could be instigated.

4.1 Isolation of a Bacterial Contaminant in Bottled Milk

The first objective of this research was to identify bacterial species that contaminate bottled milk. Trim milk was chosen due to the higher level of consumer complaints about this type of milk. In addition, trim milk contains a higher concentration of protein than other types of milk which attracts protease producing bacteria (Deeth *et al.*, 2002). It is thought that a combination of high bacterial population and high hydrolytic enzyme production causes an increased rate of organoleptic changes in milk (Dogan and Boor, 2003). Bacterial populations of trim milk were followed over 10 days when incubated at 7°C. This temperature was chosen because it closely matches the temperature of domestic refrigerators and means that consumer temperature abuse of bottled milk can be simulated.

A total of 162 colonies were isolated from bottled milk and of these 55% were identified as possible pseudomonads. A further 39 colonies were found to produce protease and 23 were identified as *Pseudomonas spp* by the API 20NE identification system. Of the colonies identified, 17 were found to be *Ps. putida* and consisted of eight different API profiles or biotypes. In addition, 6 colonies were identified as *Ps. fluorescens,* they consisted of five different biotypes. This study found pseudomonads to be prevalent in milk contamination; *Ps. putida* and *Ps. fluorescens* were identified in particular. This high presence of pseudomonads in milk had been well documented in past research. In a recent study by Dogan and Boor (2003) 338 pseudomonads were isolated from raw milk, pasteurised milk and environmental swabs. The API 20NE identification system used identified 51% of colonies as *Ps. fluorescens* and 14% as *Ps. putida,* however, these percentages may have been considerably higher as 25% of colonies were identified as either *Ps. fluorescens* or *Ps. putida* and 10% of colonies were unable to be identified.

Ps. putida isolated in this research was found to have high protease activity, however, in many other studies this was not the case (Wiedmann *et al.*, 2000). In the study by Dogan and Boor (2003) 69% of *Ps. fluorescens* strains isolated produced protease compared to only 13% of *Ps. putida* strains.

A number of different *Bacillus spp* were also isolated from bottled milk and included *B. licheniformis, B. subtilis* and *B. cereus. Bacillus spp* are commonly isolated from pasteurised milk when *Pseudomonas spp* are not present or in low numbers (Cromie, 1994). This is due to their longer lag phase and generation time compared to pseudomonads (Meer *et al.*, 1991) as also conferred in this study. *B. licheniformis* and *B. subtilis* were isolated from bottled milk on day one and day four respectively during the lag phase in the bacterial population growth when colony numbers were below $6.4x10^3$ CFU/ml and pseudomonads constituted only 57% of the population. *B. cereus* was isolated from bottled milk that contained no *Pseudomonas spp.*

4.2 Tracking of Bacterial Contamination in the Milk-Processing Factory

The second objective in this research was to determine the source of bacterial contamination by *Pseudomonas* and *Bacillus spp.* To do this a number of points along the milk process line were chosen and systematically sampled. The first site sampled was immediately after pasteurisation. These homogenised milk samples were incubated for 28 days at 7°C in order to determine bacterial contamination over time. This established whether pseudomonads survived pasteurisation but were present at numbers too low to be isolated by the standard plate count method used. Homogenised milk was used because of availability, however, this research shows that there are no differences in either the population size or type of bacteria isolated from this milk compared to trim. The bacteria isolated from newly pasteurised milk were entirely Gram-positive rods and were identified as *B. cereus*, *B. circulans* and a *Corynebacterium sp*; there were no *Pseudomonas spp* isolated from this milk even after 28 day. This suggests that pseudomonads do not survive pasteurisation and therefore they must be post-pasteurisation contaminants. Alternatively, *Bacillus spp* are shown to survive pasteurisation to contaminate packaged milk.

Since pseudomonads were shown not to survive pasteurisation then contamination must have occurred after this point. The pasteurised milk storage tanks were the next possible point of contamination and hence were sampled. These milk samples were incubated at 7°C for 38 days and the types of bacteria present identified. On day 38, milk was found to contain high bacterial populations of a Gram-negative ox+ rod, which was identified at *Sphingomonas paucimobilis* (formally known as *Ps. paucimobilis*). *Ps. fluorescens* or *Ps. putida* were not isolated from tank milk. In addition to the tank milk sampled, three commercially filled bottles of milk from the same milk batch were incubated at 7°C for ten days. These bottles were found to contain high populations of *Ps. fluorescens* and *Ps. putida* which indicating that if these pseudomonads were present in tank milk they would have been isolated.

The previous two points sampled showed that if *Ps. fluorescens* and *Ps. putida* bacteria were present in the milk sampled, then they would have been detected after ten days incubation at 7°C, therefore the remaining points sampled were incubated for only ten days at 7°C. The pre-filler milk was the next point to be sampled; this milk contained a mixture of Gram-positive cocci and Gram-positive rods, there were no pseudomonads isolated. In addition to pre-filler milk, commercially filled bottles of milk were also sampled and were found to contain *Ps. fluorescens*. This suggested that the milk process line was not the source of contamination.

In past research the bottle filler and specifically the filler rubbers have been identified as key points of bacterial contamination (Murphy *et al.,* 1998). Here the physical condition of the filler rubbers was examined, as well as sampling milk from inside the filler rubbers. The used filler rubber examined was removed during a routine replacement, done every six months. When compared to a new filler rubber it was substantially worn down over the entire milk contact surface and when flexed in the same manner as normal operation, deep cracks appeared. When examined further under SEM the highly porous nature of the rubber surface was observed. During the cleaning of these filler rubbers they are not flexed in any way and the rubber swells. Therefore, it is possible that bacteria enter these cracks when flexed and remain during the cleaning process to re-contaminate milk in the next processing cycle.

Milk samples from inside the filler rubber were removed by injecting a syringe through the sides of the rubber to withdraw the samples. The sampling method itself was considered a possible point of contamination as it has been shown that bacterial contamination can enter milk via splashing from high pressure hoses into hairline or pinhole cracks in plates in the process line (Bishop and White, 1986). Despite the controversial sampling method used, there were no pseudomonads isolated from the milk samples taken.

Since pseudomonad contamination was not seen in the bottle filler other potential external sources of contamination were investigated. The chlorinated water supplying the

CIP system and the bottle washer were the first areas sampled. There were six bacterial colonies isolated from this water and only one colony, isolated from the CIP system, was identified as a Gram-negative ox+ rod. This bacterium was identified as either *Sphingomonas spiritvorum* or *Chryseobacterium meningosepticum*. This result is consistent with other studies conducted on milk factory water supplies, where no pseudomonads were isolated (Eneroth *et al.*, 2000).

Recycled bottles used by this milk-processing factory were tested for the presence of pseudomonads by comparing the bacterial population of a standard recycled bottle to that of a laboratory sterilised bottle. *Ps. fluorescens* and *Ps. putida* were found in both types of bottle and although some of the bacterial populations of the standard recycled bottles were slightly higher than those of the sterilised bottles, standard error determinations revealed that there was no difference between the two types of collection methods. This suggests that pseudomonad contamination is present in the milk before it enters the bottle.

A common source of pseudomonad contamination is condensed water collecting on filler parts and aerosols within the bottle filler room (Eneroth *et al.*, 2000, Chadwich-hayes and Boor, 2001). A number of environmental swabs were taken from surfaces around the bottling room. The swabs taken from the factory worker stairs were the only swabs that contained high levels of *Ps. fluorescens*. Other swabs were found to contain species of *Aeromonas*, *Burkholderia* and *Chryseobacterium spp*. This result confirms the presence of pseudomonads in the bottling room environment and hence a possible origin for *Pseudomonas spp* contamination. In many other studies conducted, gross contamination by *Ps. fluorescens* has also been isolated from the bottling room environment (Dogan and Boor, 2003, Murphy *et al.*, 1998).

4.3 Seasonal Variations in Bacterial Contamination of Milk

The third objective in this study was to identify seasonal variation in the type of bacteria contaminating milk. Bacterial populations in milk were initially sampled from various

sites throughout the milk-processing factory in the summer and these bacterial populations were compared to those found in winter milk. Raw milk contained the highest seasonal changes in bacterial population from all the sites sampled. The bacterial population was $1.6x10^7$ CFU/ml higher in winter milk compared to that found in summer milk. The types of bacteria present in winter milk compared to summer milk was also very different with winter milk dominated by pseudomonads and summer milk containing largely Gram-positive cocci. In a study by Fischer *et al.* (1987) a similar result was reported with an increase in *Pseudomons spp* in winter and an increase in Gram-positive cocci, such as lactic streptococci, in the summer. There has also been work conflicting with these findings. Andrey and Frazier (1959) found a seven-fold increase in bacterial populations when cows were put out to pasture in spring. Of the species isolated in this study, *Flavobacterium sp*, a Gram-negative ox- rod, dominated bacterial populations. Uraz and Çitak (1998) found the highest proportion of *Pseudomonas spp* isolated from raw milk were in the spring and summer with two and four times higher populations than those isolated in winter.

The bacterial populations of the other milk samples taken were very similar in population size between the seasons, however, there were a few changes in the types of bacteria present in the milk samples. The newly pasteurised milk samples obtained in the summer contained mostly Gram-positive cocci, whereas in the winter the bacterial populations consisted of mostly Gram-positive rods. Bacterial populations isolated from glass bottled milk were dominated by Gram-positive rods in the winter and Gram-negative ox- rods in the summer. The remaining samples of milk obtained from the pasteurised milk storage tanks, plastic bottles, sachets and cartons all contained high populations of Gram-positive rods in both seasons.

The high proportion of Gram-positive rods found in pasteurised milk, especially during winter, may be due to the higher levels of bacterial spores found in raw milk at this time of year (Meer *et al.*, 1991). When pseudomonad populations are eliminated by pasteurisation these spores are able to germinate and grow. In summer milk, however, Gram-positive cocci, such as *Micrococcus spp* dominate raw milk (Kikuchi and Matsui,

1976). These bacteria are also thermoduric psychrotrophs and are able to survive pasteurisation to contaminate pasteurised milk (Cousin, 1982).

Types of bacteria contained in bottled milk (incubated for ten days at 7°C) were also compared between the seasons. In early summer, mid-autumn and late autumn bacterial populations of *Ps. fluorescens* and *Ps. putida* were considerably high with 17 of the 18 bottles sampled exceeding $5.0x10^5$ CFU/ml. In mid-spring, however, Gram-positive cocci dominated the bacterial populations and only one of the six bottles sampled exceeded $5.0x10^5$ CFU/ml.

The change in the types of bacteria contained in milk may be explained by the changes in the supply of milk to the milk-processing factory. During this research the supply location of raw milk changed. From January to June raw milk was obtained from the Canterbury region, however, in the months of June to July there were shortages of milk in Canterbury and milk was freighted by rail from the North Island. This meant that raw milk was held for up to four days at refrigeration temperatures before being pasteurised. In early August supply was changed back to the Canterbury region and remained this way until the completion of the research.

4.4 Summary

This study established the presence of *Pseudomonas spp* and *Bacillus spp* in milk from a milk-processing factory. The growth of these psychrotrophic bacteria and hence production of hydrolytic enzymes caused unfavorable organoleptic traits to develop in the milk. The presence of *Bacillus spp* is a result of their heat resistant spores surviving pasteurisation, whereas, *Pseudomonas spp* were most likely from an environmental source. Contamination from the bottle filler, however, can not be excluded. Molecular tracking of *Pseudomonas spp* would afford some potential in reaching a definitive conclusion. Seasonal variations in contaminating *Bacillus* and *Pseudomonas spp* did not follow reported trends (Cousin, 1982, Meer *et al.*, 1991). The population of *Bacillus spp* over the winter and *Pseudomonas spp* in the spring both decreased.

CHAPTER V

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APPENDIX I

MEDIA

Luria Beritani Medium (LB)

10g	Tryptone (Oxoid)
5g	Yeast extract (Gibco)
5g	Sodium chloride
15g	Agar (Oxoid)
1000ml	Distilled or deionised water

Dissolve tryptone, yeast extract and sodium chloride in 950ml of distilled water. Adjust solution to pH 7.2± 0.2 at 25°C with 1*N* NaOH and makeup to 1000ml. Add agar and autoclave for 20 min at 121°C and 103.4 kPa.
(Atlas, 1993)

Milk Plate Count Agar (MPCA)

5g	Tryptone (Oxoid)
2.5g	Yeast extract (Gibco)
1g	Glucose monohydrate
1g	Skimmed milk powder (Mainland)
15g	Agar (Oxoid)
1000ml	Distilled or deionised water

Dissolve all ingredients (except agar) in 950ml distilled water. Adjust solution to pH 7.0± 0.2 at 25°C and makeup to 1000ml. Add agar and autoclave as described above.
(Bridson, 1990)

Milk Agar

5.63g Plate Count Agar (Oxoid)
5g Skim milk powder (Mainland)
300ml Distilled or deionised water

Dissolve skim milk powder in 50ml water and autoclave for 10 min (at 121°C and 103.4kPa). Add plate count agar to 250ml of water and autoclave for 20 min. Mix two solutions and pour immediately.
(Atlas, 1993)

Goulds Medium

18g Agar (Oxoid)
10g Sucrose
10ml Glycerol
5g Casamino acids (hydrolysed extract of casein) (Difco)
1g $NaHCO_3$
1.76g $K_2HPO_4.3H_2O$
1g $MgSO_4.7H_2O$
1.2g Sodium laurol sarcosine
20mg Trimethoprim lactate or Trimethoprim
1000ml Distilled or deionised water

Solution A: dissolve sucrose, glycerol, casamino acids, $NaHCO_3$, and $K_2HPO_4.3H_2O$ in 500ml of water and autoclave. Solution B: add agar to 500ml of water and autoclave. Stock solutions include filter sterilised sodium laurol sarcosine stock solution (2.4g dissolved in 10ml of water); autoclaved 1M $MgSO_4.7H_2O$ stock solution (24.6g dissolved in 100ml of water) and non-sterilised trimethoprim (30mg dissolved in 3ml of methanol). Add 5ml of sodium laurol sarcosine, 40µl of $MgSO_4.7H_2O$ and 2ml of trimethoprim to

agar when cooled 45°C. Combine solutions A and B and store at room temperature. (Reid, 1997)

LB Storage Broth

0.5g Tryptone (Oxoid)
0.25g Yeast extract (Gibco)
0.25g Sodium chloride
7.5ml Glycerol
42.5ml Distilled or deionised water

Add all components, adjust to pH to 7.2± 0.2 at 25°C and autoclave.

Peptone Water

10g Peptone (Oxide)
5g Sodium chloride
1000ml Distilled or deionised water

Add all components, adjust pH to 7.2± 0.2 at 25°C and autoclave.
(Atlas, 1993)

APPENDIX II

SUPPLEMENTARY FIGURES AND TABLES

Table AII.1: *API 20NE Biotypes*

Bacterial species	API Code	Biotype	Bacterial Species	API Code	Biotype
Ps. fluorescens	O157555	1f	*Ps. putida*	4353455	1p
Ps. fluorescens	1353455	2f	*Ps. putida*	4141455	2p
Ps. fluorescens	O156575	3f	*Ps. putida*	O143455	3p
Ps. fluorescens	O357555	4f	*Ps. putida*	O141455	4p
Ps. fluorescens	O157575	5f	*Ps. putida*	4343455	5p
Ps. fluorescens	O346455	6f	*Ps. putida*	4143455	6p
Ps. fluorescens	O153455	7f	*Ps. putida*	O343455	7p
			Ps. putida	O143445	8p

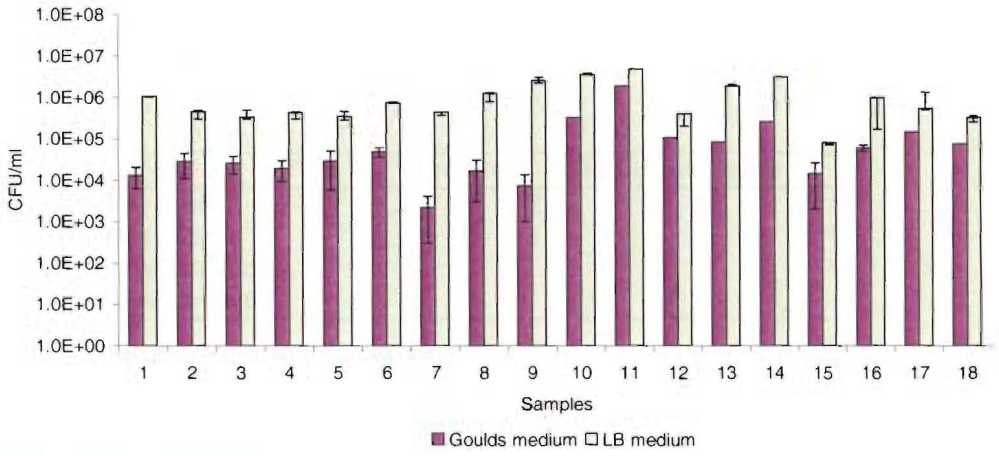

Figure AII.1: *Bacterial Population of Trim Milk on Expiry Date. These 18 bottles were incubated for ten days at 7°C and then 100µl samples were spread onto two different media, Goulds and LB medium. The error bars were calculated from the standard deviation of the mean.*

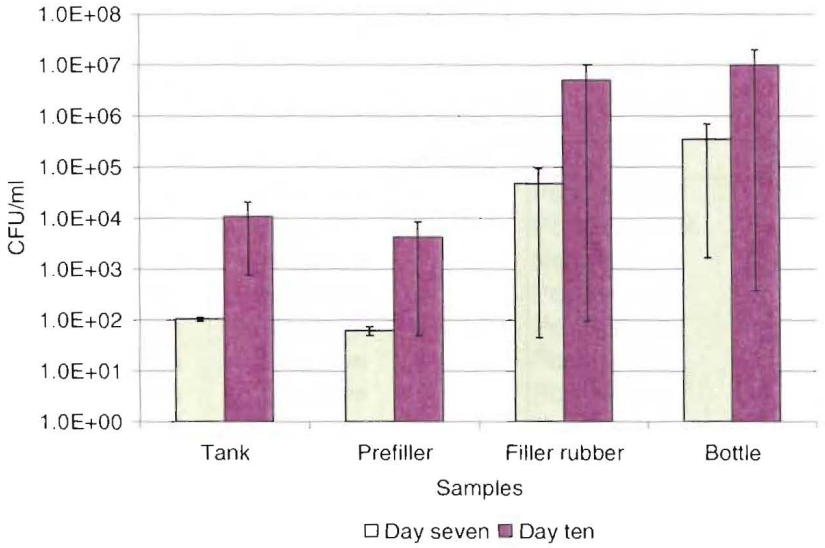

Figure AII.2: *The Bacterial Population of Tank, Pre-filler, Filler Rubber and Bottled Milk. These samples were incubated at 7°C for ten days. 100µl samples were removed from each sample on days seven and ten and were plated onto LB medium. The error bars were calculated from the standard deviation of the mean.*

Table AII.2: *Isolates Identified From Tank, Pre-filler, Filler Rubber and Bottled Milk. These colonies were isolated on day ten.*

Types of Bacteria	Tank	Pre-filler	Filler Rubber	Bottle
Gram-positive rods	3	2	2	1
Gram-positive cocci		1	2	1
Gram-negative ox+ rods				1
Gram-negative ox- rods				

Table AII.3: *The Bacterial Population of Environmental Swabs. Counts were recorded in total CFU isolated.*

Swab sites	Swab 1	Swab 2	Average	STER
Filler head	5.00E+00	1.40E+01	9.50E+00	4.50E+00
Filler bowl	2.30E+03	6.80E+02	1.49E+03	8.10E+02
Steps	3.44E+02	2.85E+04	1.44E+04	1.41E+04
Drain	3.70E+01	4.10E+01	3.90E+01	2.00E+00
Transfer wheel	1.00E+00		1.00E+00	

APPENDIX III

RAW DATA

Table AIII.1: *Bacterial Isolate Descriptions from Bottled Trim Milk over Ten Days.*

Day	Bottle #	Isolate	Protease	Oxidase	Gram Stain	Identification
0	7	a	Negative		Positive rod	
0	2	b	Negative		Positive cocci	
0	1	d	Negative		Negative rod	
0	4	f	Negative		Positive rod	
0	8	h	Negative		Positive rod	
1	10	a	Negative		Positive rod	
1	10	c	Negative		Positive rod	
1	5	d	Negative		Positive rod	
1	9	e	Negative		Negative rod	
1	6	h	Positive		Positive rod	*B. licheniformis*
2	8	a	Negative		Positive rod	
2	8	b	Negative		Positive rod	
2	3	d	Negative		Negative rod	
2	2	e	Negative		Positive rod	
2	2	f	Negative		Positive rod	
3	7	a	Positive	Positive	Negative rod	*Ps. putida*
3	2	b	Positive	Positive	Negative rod	*Ps. putida*
3	1	c	Negative		Positive cocci	
3	1	d	Negative		Negative rod	
3	3	e	Negative		Positive cocci	
3	8	h	Positive		Positive rod	
3	10	g	Negative	Positive	Negative rod	*Ps. fluorescens*
3	4	f	Positive		Positive rod	
4	10	a	Negative		Negative rod	
4	7	b	Positive		Positive rod	
4	2	c	Positive		Positive rod	*B. subtilus*
4	2	e	Positive		Positive rod	*Bacillus species*
4	2	g	Negative		Negative rod	
4	1	j	Negative		Negative rod	
4	6	l	Negative		Negative rod	
5	7	b	Negative		Negative rod	
5	5	e	Negative		Negative rod	
6	6	a	Negative		Negative rod	
6	9	d	Negative	Positive	Negative rod	*Ps. fluorescens*
6	8	e	Positive	Positive	Negative rod	*Ps. putida*
6	7	f	Positive	Positive	Negative rod	*Ps. putida*
7	5	a	Negative	Positive	Negative rod	
7	9	d	Negative	Positive	Negative rod	
7	9	e	Negative	Positive	Negative rod	

Day	Bottle #	Isolate	Protease	Oxidase	Gram Stain	Identification
8	6	c	Negative	Positive	Negative rod	
8	6	d	Negative	Positive	Negative rod	*Ps. putida*
8	10	e	Negative	Positive	Negative rod	*Ps. putida*
8	1	g	Negative	Positive	Negative rod	
9	10	a	Negative	Positive	Negative rod	
9	1	c	Negative	Positive	Negative rod	
9	7	e	Negative	Positive	Negative rod	*Ps. putida*
9	3	f	Negative		Positive cocci	
10	7	a	Negative		Positive rod	
10	10	b	Negative		Positive rod	
10	10	c	Negative		Positive rod	
10	10	d	Negative		Positive rod	
10	5	e	Negative		Positive rod	
10	1	f	Positive	Positive	Negative rod	*Ps. fluorescens*

Table AIII.2: *Bacterial Isolate Descriptions from Bottled Trim Milk on Expiry Date.*

Date	Colony #	Media	Protease	Oxidase	Gram stain	Identification
2-May	1a	LB	Negative	Positive	Negative rod	
	1b	LB	Positive	Positive	Negative rod	*Ps. putida*
	1f	LB	Negative	Positive	Negative rod	
	1a	G	Positive		Positive rod	
	1b	G	Negative		Positive rod	
	2a	LB	Positive	Positive	Negative rod	*Ps. putida*
	2b	LB	Negative		Positive rod	
	2a	G	Positive		Positive rod	
	2b	G	Positive		Positive rod	
	2c	G	Negative	Positive	Negative rod	
	3a	LB	Negative	Positive	Negative rod	
	3b	LB	Negative		positive cocci	
	3c	LB	Positive	Positive	Negative rod	
	3d	LB	Positive	Positive	Negative rod	
	3e	LB	Negative		Positive rod	
	3a	G	Negative	Positive	Negative rod	
	3b	G	Positive		Positive rod	
	3e	G	Positive	Positive	Negative rod	
	4a	LB	Negative		positive cocci	
	4b	LB	Negative	Positive	Negative rod	
	4c	LB	Positive	Positive	Negative rod	
	4a	G	Negative	Positive	Negative rod	
	4b	G	Positive	Negative	Negative rod	
	5a	LB	Negative		Positive rod	
	5b	LB	Negative	Positive	Negative rod	
	5a	G	Negative	Negative	Negative rod	
	5d	G	Negative	Negative	Negative rod	
3-May	1a	LB	Positive	Positive	Negative rod	*Ps. putida*
	1b	LB	Negative	Positive	Negative rod	
	1c	LB	Negative	Positive	Negative rod	

Date	Colony #	Media	Protease	Oxidase	Gram stain	Identification
	1a	G	Positive	Positive	Negative rod	
	1b	G	Negative	Negative	Negative rod	
	1c	G	Positive	Positive	Negative rod	*Ps. putida*
	2a	LB	Positive	Positive	Negative rod	
	2b	LB	Negative	Negative	Negative rod	
	2d	LB	Positive	Positive	Negative rod	
	2e	LB	Negative	Positive	Negative rod	
	2a	G	Negative	Positive	Negative rod	
	2b	G	Positive	Positive	Negative rod	
	2c	G	Positive		Positive cocci	
	3a	LB	Negative	Positive	Negative rod	
	3b	LB	Positive	Positive	Negative rod	
	3a	G	Negative	Positive	Negative rod	
	3b	G	Positive	Positive	Negative rod	
	4a	LB	Negative		Positive cocci	
	4b	LB	Negative	Positive	Negative rod	
	4c	LB	Negative	Positive	Negative rod	
	4d	LB	Negative	Positive	Negative rod	
	4e	LB	Negative	Positive	Negative rod	
	4a	G	Positive		Positive rod	
	4c	G	Positive	Positive	Negative rod	
	4d	G	Positive	Negative	Negative rod	
	4e	G	Positive	Positive	Negative rod	*Ps. fluorescens*
4-May	1a	LB	Negative	Negative	Negative rod	
	1b	LB	Negative		Positive rod	
	1c	LB	Positive	Positive	Negative rod	*Ps. fluorescens*
	1d	LB	Negative	Negative	Negative rod	
	1a	G	Positive	Positive	Negative rod	*Ps. putida*
	1b	G	Positive	Negative	Negative rod	
	1c	G	Positive		Positive rod	
	2a	LB	Positive	Negative	Negative rod	
	2b	LB	Negative	Positive	Negative rod	
	2c	LB	Negative		Positive rod	
	2a	G	Negative	Positive	Negative rod	
	2b	G	Positive	Positive	Negative rod	
	3a	LB	Negative	Positive	Negative rod	
	3b	LB	Positive	Positive	Negative rod	
	3c	LB	Positive	Positive	Negative rod	
	3d	LB	Negative		Positive rod	
	3a	G	Positive	Positive	Negative rod	
	3b	G	Positive		Positive rod	
	3d	G	Negative		Positive rod	
	4a	LB	Negative	Positive	Negative rod	
	4b	LB	Negative	Negative	Negative rod	
	4d	LB	Negative	Negative	Negative rod	
	4a	G	Positive		Positive rod	

	Colony #	Media	Protease	Oxidase	Gram stain	Identification
	4b	G	Negative	Positive	Negative rod	
	4c	G	Negative		Positive rod	
	4d	G	Positive	Positive	Negative rod	*Ps. putida*
	4e	G	Positive	Positive	Negative rod	*Ps. fluorescens*
	5b	LB	Negative		Positive rod	
	5c	LB	Negative		Positive rod	
	5d	LB	Negative		Positive rod	
	5a	G	Positive	Positive	Negative rod	
	5b	G	Positive	Positive	Negative rod	
5-May	1a	LB	Positive	Positive	Negative rod	*Ps. putida*
	1c	LB	Positive	Positive	Negative rod	*Ps. putida*
	1d	LB	Positive	Positive	Negative rod	
	1a	G	Positive	Positive	Negative rod	
	1c	G	Positive	Positive	Negative rod	*Ps. putida*
	1d	G	Positive	Positive	Negative rod	
	2a	LB	Negative		Positive cocci	
	2b	LB	Negative	Positive	Negative rod	
	2d	LB	Negative		Positive cocci	
	2e	LB	Negative		Positive cocci	
	2f	LB	Positive		Positive rod	
	2a	G	Negative	Negative	Negative rod	
	2b	G	Positive	Positive	Negative rod	*Ps. putida*
	2c	G	Negative	Positive	Negative rod	
	3a	LB	Negative	Positive	Negative rod	
	3b	LB	Negative	Positive	Negative rod	
	3a	G	Positive	Positive	Negative rod	
	3b	G	Negative	Negative	Negative rod	
	4a	LB	Negative	Positive	Negative rod	
	4b	LB	Negative	Negative	Negative rod	
	4a	G	Positive		Positive rod	

Table AIII.3: *The Bacterial Population of Bottled Trim Milk in CFU/ml: data pertaining to Figure 3.1.*

Days	Bottle 1	Bottle 2	Bottle 3	Bottle 4	Bottle 5	Bottle 6	Bottle 7	Bottle 8	Bottle 9	Bottle 10	Average	STER
0	1.70E+02	1.80E+02	2.20E+02	2.20E+02	1.90E+02	2.10E+02	1.00E+02	1.40E+02	2.00E+02	2.20E+02	1.85E+02	1.24E+01
1	1.30E+02	1.00E+02	1.20E+02	1.20E+02	1.30E+02	1.40E+02	1.40E+02	2.40E+02	1.70E+02	1.80E+02	1.47E+02	1.27E+01
2	3.60E+02	2.10E+02	3.10E+02	2.40E+02	1.70E+02	2.80E+02	2.20E+02	2.20E+02	2.30E+02	2.10E+02	2.45E+02	1.77E+01
3	1.40E+03	2.70E+02	3.80E+02	2.90E+02	3.10E+02	1.90E+02	2.60E+02	3.30E+02	4.90E+02	2.20E+02	4.14E+02	1.13E+03
4	1.18E+04	9.20E+02	2.57E+03	1.25E+04	9.60E+03	1.58E+04	1.47E+03	1.92E+03	2.36E+03	5.40E+03	6.43E+03	1.74E+04
5	1.62E+04	6.92E+03	3.13E+04	2.48E+04	3.35E+04	5.30E+04	8.58E+03	1.60E+04	6.36E+03	1.18E+04	2.08E+04	4.71E+04
6	3.84E+06	1.71E+06	1.69E+06	4.75E+06	5.34E+06	9.08E+06	1.74E+06	1.89E+06	7.60E+05	1.30E+06	3.21E+06	8.16E+05
7	4.10E+06	1.12E+07	3.50E+06	6.10E+06	5.70E+06	6.10E+06	3.80E+06	4.70E+06	7.80E+06	3.10E+06	5.61E+06	7.72E+05
8	1.60E+07	3.10E+07	1.90E+07	8.00E+06	6.00E+06	2.00E+07	1.00E+07	6.00E+06	1.00E+07	2.00E+06	1.28E+07	2.75E+06
9	8.00E+06	1.00E+06	9.00E+06	1.00E+06	7.00E+06	4.00E+06	5.00E+06	4.00E+06	3.00E+06	2.50E+07	6.7E+06	2.21E+06
10	5.20E+06	1.10E+07	5.50E+06	8.90E+06	3.60E+07	3.90E+06	1.45E+08	2.32E+07	2.50E+06	7.00E+06	2.48E+07	1.38E+07

Table AIII.4: *Bacterial Populations of Bottled Trim and Homogenised Milk in CFU/ml: Data pertaining to Figure 3.3*

Days	Bottle 1	Bottle 2	Bottle 3	Average	STER	Days	Bottle 1	Bottle 2	Bottle 3	Average	STER
0	1.80E+02	1.40E+02	1.50E+02	1.57E+02	1.20E+01	0	9.00E+01	1.10E+02	1.20E+02	1.07E+02	8.82E+00
1	1.70E+02	1.50E+02		1.60E+02	1.00E+01	1	1.10E+02	9.00E+01		1.00E+02	1.00E+01
2	1.60E+02	1.40E+02		1.50E+02	1.00E+01	2	5.00E+01	1.30E+02		9.00E+01	4.00E+01
5	5.00E+01	7.00E+01		6.00E+01	1.00E+01	5	5.79E+02	1.40E+02		3.60E+02	2.20E+02
6	3.70E+02	1.30E+02		2.50E+02	1.20E+02	6	1.91E+03	5.50E+02		1.23E+03	6.80E+02
7	1.90E+03	8.70E+02		1.39E+03	5.15E+02	7	4.70E+04	4.20E+03		2.56E+04	2.14E+04
8	1.45E+04	1.00E+03		7.75E+03	6.75E+03	8	3.90E+05	1.70E+04		2.04E+05	1.87E+05
11	2.21E+05	6.80E+03	3.60E+04	8.79E+04	6.71E+04	11	1.34E+06	3.16E+05	1.20E+05	5.92E+05	3.78E+05

Table AIII.5: *The Bacterial Population of Newly Pasteurised Milk in CFU/ml: data pertaining to Figure 3.5*

Days	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Average	STER
0	1.00E+02	1.00E+02	1.20E+02	1.00E+02	1.10E+02	1.06E+02	4.00E+00
4	6.00E+01	5.00E+01	8.00E+01	9.00E+01	4.00E+01	6.40E+01	9.27E+00
6	9.00E+01	1.00E+02	8.00E+01	1.30E+02	1.30E+02	1.06E+02	1.03E+01
8	9.00E+01	1.20E+02	1.00E+02	8.00E+01	7.00E+01	9.20E+01	8.60E+00
10	8.00E+01	1.40E+02	9.00E+01	8.00E+01	6.00E+01	9.00E+01	1.34E+01
13	7.00E+01	6.00E+01	2.60E+02	4.10E+02	1.30E+02	1.86E+02	6.64E+01
18	2.88E+03	8.00E+01	1.00E+02	7.16E+03	1.84E+03	2.41E+03	1.30E+03
20	1.09E+04	2.10E+02	1.10E+02	2.69E+04	2.90E+03	8.20E+03	5.07E+03
22	5.20E+04			7.10E+04	2.59E+04	4.96E+04	1.31E+04
26	7.00E+04	4.90E+04		5.00E+04	2.00E+04	4.70E+04	1.03E+04
28	1.60E+05	2.80E+04		1.00E+05	1.03E+06	3.30E+05	2.35E+05

Table AIII.6 a, b and c: *The Bacterial Population of Tank Milk, Standard Bottle Milk and Sterile Bottle Milk: data pertaining to Figures 3.7 and 3.11*

Table AIII.6a. *Tank milk, data in CFU/ml*

Days	Sample 1	Sample 2	Sample 3	Average	STER
0	2.30E+02	1.70E+02	4.00E+02	2.67E+02	6.89E+01
2	3.40E+02	3.10E+02	2.50E+02	3.00E+02	2.65E+01
4	3.20E+02	4.50E+02	2.00E+02	3.23E+02	7.22E+01
8	5.90E+02	1.83E+03	4.20E+02	9.47E+02	4.44E+02
10	2.40E+03	9.20E+02		1.66E+03	7.40E+02
38	3.02E+05	2.85E+06	1.16E+06	1.44E+06	7.49E+05

Table AIII.6b. *Standard bottle milk, data in CFU/ml*

Days	Bottle 1	Bottle 2	Bottle 3	Average	STER
0	3.10E+02	3.09E+02	1.80E+02	2.66E+02	4.32E+01
2	2.70E+02	2.80E+02	3.90E+02	3.13E+02	3.84E+01
4	1.90E+02	2.80E+02	2.20E+02	2.30E+02	2.65E+01
10	2.87E+07	3.00E+06	3.40E+06	1.17E+07	8.50E+06

Table AIII.6c. *Sterile bottle, data in CFU/ml*

Days	Bottle 1	Bottle 2	Bottle 3	Average	STER
0	2.20E+02	4.10E+02	2.10E+02	2.80E+02	6.51E+01
2	3.30E+02	3.10E+02	3.30E+02	3.23E+02	6.67E+00
4	3.20E+02	1.70E+02	2.30E+02	2.40E+02	4.36E+01
10	2.08E+06	2.04E+06	4.36E+06	2.83E+06	7.67E+05

Table AIII.7a: *The Bacterial Population of Summer Milk Isolated on MPCA Medium in CFU/ml: data pertaining to 3.13 b.*

Sample site	Raw	Past.	Tank	Bottle	Plastic	Sachet	Carton
1	2.30E+03	1.80E+02	1.10E+02	1.30E+02	2.30E+02	1.60E+02	1.50E+02
2	2.00E+03	2.60E+02	1.10E+02	1.30E+02	1.80E+02	2.40E+02	1.40E+02
3	1.80E+03	2.70E+02	1.50E+02	1.80E+02	2.30E+02	1.50E+02	2.40E+02
4	3.00E+03	1.20E+02	1.20E+02	1.90E+02	1.10E+02	2.30E+02	1.20E+02
5		2.50E+02	1.10E+02	1.80E+02	1.20E+02	2.10E+02	1.70E+02
6		1.10E+02	8.00E+01				
7		2.10E+02	1.50E+02				
8		4.90E+02	1.40E+02				
9		2.30E+02	2.00E+01				
10		1.60E+02	4.00E+01				
Average	2.28E+03	2.39E+02	1.11E+02	1.62E+02	1.74E+02	1.98E+02	1.64E+02
STER	2.63E+02	3.41E+01	1.40E+01	1.32E+01	2.58E+01	1.83E+01	2.06E+01

Table AIII.7b: *The Bacterial Population of Summer Milk Isolated on LB Medium in CFU/ml: data pertaining to 3.13 b.*

Sample site	Raw	Past.	Tank	Bottle	Plastic	Sachet	Carton
1	2.50E+03	5.00E+01	4.00E+01	2.90E+02	1.10E+02	9.00E+01	1.10E+02
2	9.00E+02	1.10E+02	7.00E+01	7.00E+01	9.00E+01	1.70E+02	1.50E+02
3	2.00E+03	4.00E+01	3.00E+01	6.00E+01	1.00E+01	6.00E+01	2.20E+02
4		2.00E+01	1.60E+02	8.00E+01	3.00E+01	2.40E+02	1.60E+02
5		5.00E+01	2.00E+01	2.40E+02	5.00E+01	5.00E+01	8.00E+01
6		1.80E+02	2.00E+01				
7		2.00E+01	3.00E+01				
8		1.70E+02	1.20E+02				
9		1.60E+02					
10							
Average	1.80E+03	9.80E+01	5.10E+01	1.48E+02	5.80E+01	1.22E+02	1.44E+02
STER	4.73E+02	2.17E+01	1.67E+01	4.85E+01	1.85E+01	3.62E+01	2.38E+01

Table AIII.8a: *The Bacterial Population of Winter Milk Isolated on MPCA Medium in CFU/ml: data pertaining to 3.13 a*

Sample site		Raw	Past.	Tank	Bottle	Plastic	Sachet	Carton
Sample	1	1.00E+05	7.00E+01	9.00E+01	9.00E+01	2.00E+01	7.00E+01	1.10E+02
Sample	2	3.10E+07		2.00E+02			2.00E+02	2.00E+02
Average		1.56E+07	7.00E+01	1.45E+02	9.00E+01	2.00E+01	1.35E+02	1.55E+02
STER		1.55E+07	0.00E+00	5.50E+01	0.00E+00	0.00E+00	6.50E+01	4.50E+01

Table AIII.8b: *The Bacterial Population of Summer Milk Isolated on LB Medium in CFU/ml: data pertaining to 3.13 a*

Sample site		Raw	Past.	Tank	Bottle	Plastic	Sachet	Carton
Sample	1	4.00E+04	1.20E+02	1.20E+02	2.00E+02	1.30E+02	2.30E+02	5.00E+01
Sample	2	4.00E+05		1.00E+02	5.00E+02	2.00E+02		3.00E+01
Average		2.20E+05	1.20E+02	1.10E+02	3.50E+02	1.65E+02	2.30E+02	4.00E+01
STER		1.80E+05	0.00E+00	1.00E+01	1.50E+02	3.50E+01	0.00E+00	1.00E+01